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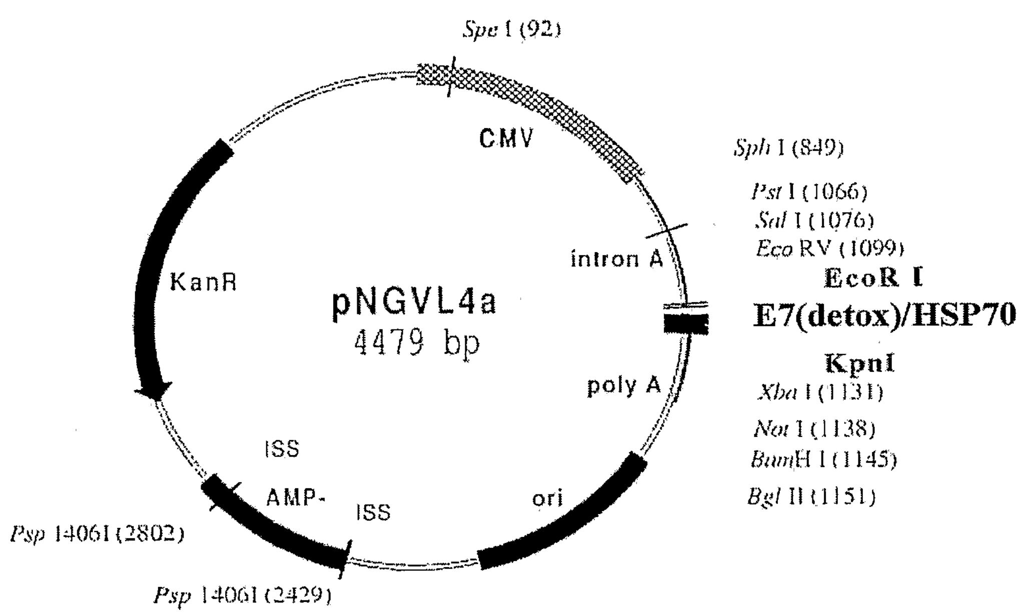
(54) Title: ANTI-CANCER DNA VACCINE EMPLOYING PLASMIDS ENCODING SIGNAL SEQUENCE, MUTANT ONCOPROTEIN ANTIGEN, AND HEAT SHOCK PROTEIN



(57) Abstract: Novel nucleic acid vectors comprising sequences encoding (a) an antigen, (b) a signal peptide, and (c) a heat shock protein, are disclosed, as are methods for using such vectors to induce antigen-specific immune responses and to treat tumors.

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Anti-Cancer DNA Vaccine Employing Plasmids Encoding Signal Sequence, Mutant Oncoprotein Antigen, and Heat Shock Protein

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to chimeric nucleic acid molecules that encode an antigen, a signal peptide, and an immunogenicity-potentiating polypeptide ("IPP") such as the heat shock protein HSP70, and their uses a immunogenic compositions to induce and enhance immune responses, primarily cytotoxic T lymphocyte responses to specific antigens such as tumor or viral antigens.

Description of the Background Art

Cytotoxic T lymphocytes (CTL) are critical effectors of anti-viral and antitumor responses (reviewed in Chen, CH *et al.*, J Biomed Sci. *5:* 231-252, 1998; Pardoll, DM. Nat Med. *4:* 525-531, 1998; Wang, RF *et al.*, Immunol Rev. *170:* 85-100, 1999). Activated CTL are effector cells that mediate antitumor immunity by direct lysis of their target tumor cells or virus-infected cells and by releasing of cytokines that orchestrate immune and inflammatory responses that interfere with tumor growth or metastasis, or viral spread. Depletion of $CD8^+$ CTL leads to the loss of antitumor effects of several cancer vaccines (Lin, K-Y *et al.*., Canc Res. *56:* 21-26, 1996; Chen, C-H *et al.*, Canc Res. *60:* 1035-42, 2000). Therefore, the enhancement of antigen presentation through the MHC class I pathway to $CD8^+$ T cells has been a primary focus of cancer immunotherapy.

DNA vaccines have emerged as an attractive approach for antigen-specific cancer immunotherapy. DNA vaccines offer many advantages over more conventional vaccines, such as peptide or attenuated live pathogens. One advantage is that DNA vaccines are reasonably stable and can be easily prepared and harvested in large quantities. Additionally, naked plasmid DNA is relatively safe and can be repeatedly administered without adverse effects. Furthermore, because DNA is able to be maintained in cells for long-term expression of the encoded antigen, maintenance of immunologic memory is possible (for reviews, see Donnelly, JJ *et al.*, *Annu Rev Immunol* 1997, 15:617-648; Pardoll, D.M., *Nat Med* 1998, 4(5 Suppl):525-531; Robinson, HL, *Vaccine* 1997, 15:785-787; Gurunathan, S *et al.*, *Annu Rev Immunol* 2000, 18:927-974).

However, one limitation of these vaccines is their lack of potency, since the DNA vaccine vectors generally do not have the intrinsic ability to be amplified and to spread *in vivo* as do some replicating viral vaccine vectors. Furthermore, some tumor antigens such as the E7 protein of human papillomavirus-16 ("HPV-16") are weak immunogens (Chen et al., 2000, *supra*). Therefore, there is a need in the art for strategies to enhance DNA vaccine potency, particularly for more effective cancer and viral immunotherapy.

Heat Shock Proteins

Cells respond to stressors (typically heat shock) by increasing the expression of a group of genes commonly referred to as stress, or heat shock, genes. As used herein, a "heat shock protein" (abbreviated either HSP or Hsp) or "stress protein," is encoded by a stress gene, and is therefore typically produced in significantly greater amounts upon the contact or exposure of the cell or organism to the stressor. A heat shock gene is a gene that is activated or otherwise detectably upregulated as a result of stressor exposure (which may include heat shock, hypoxia, glucose deprivation, a heavy metal salt, an inhibitor of energy metabolism and electron transport, and protein denaturant, or to certain benzoquinone ansamycins. See, for example, U.S. Pat. 6,524,825 and Nover, L., *Heat Shock Response*, CRC Press, Inc., Boca Raton, Fla. (1991), both of which are hereby incorporated by reference. Stress genes includes native homologues within known stress gene families, such as certain genes within the Hsp70 and Hsp90 stress gene families, even though not every homologue is induced by a stressor.

These proteins appear to participate in important cellular processes such as protein synthesis, intracellular trafficking, and assembly and disassembly of protein complexes. The increased amounts of stress proteins synthesized during stress serve primarily to minimize the consequences of induced protein unfolding. Indeed, the preexposure of cells to mildly stressful conditions that induce stress proteins protects the cells from the deleterious effects of more extreme forms of stress. The major stress proteins appear to be expressed in every organism and tissue type) examined so far. Moreover, they represent the most highly conserved group of proteins identified to date. For example, when stress proteins in widely diverse organisms are compared. Hsp90 and Hsp70 exhibit 50% or higher identity at the amino acid level and share many similarities at non-identical positions. Similar or greater homology exists between different members of a particular stress protein family within a species.

The predominant stress proteins in bacteria have molecular masses around 70 and 60 kDa, , that are commonly referred to as Hsp70 and Hsp60, respectively. These represent about 1-3% of the total cell protein but accumulate to levels as high as 25% under stressful conditions.

Genes encoding stress proteins may be present in single or multiple, non-identical copies in a genome . For example, the human genome has at least one copy of an hsp100 gene, at least two different hsp90 genes, up to ten hsp70 genes of which at least several are non-identical copies, several T complex genes (Tcp genes) and at least one gene encoding the related mitochondrial protein Hsp60, as well as at least three copies of small hsp genes encoding Hsps of 20-30 kDa. Most families of stress genes include at least one member whose expression is relatively high and is either entirely constitutive or only mildly inducible. Furthermore, several families of stress genes include members that are not up-regulated by heat but are by other signals, *e.g.* increased calcium levels.

The stress proteins, particularly Hsp70, Hsp60, Hsp20-30 and Hsp10, are among the major determinants recognized by the immune system in response to infection by *M. tuberculosis* and *M. leprae* (Young, RA *et al.*, Cell, 1989, 50:5-8. Even healthy individuals with no history of mycobacterial infection or autoimmune disease carry T cells that recognize both bacterial and human Hsp60 epitopes; a considerable fraction of T cells expressing the γδ T cell receptor recognize both self and foreign stress proteins (O'Brien, R *et al.* Cell, 1989 57:664-674 (1989). The "system" recognizing Hsp epitopes is considered to be an "early defense system" against invading microorganisms (Murray, PJ *et al.*, J. Bacteriol. 174:4193-6 (1992)) and may be maintained by frequent stimulation by bacteria and viruses. The safety of stress proteins is demonstrated by the success and relative safety of BCG (Bacille Calmette Guerin, a strain of *M. bovis*) vaccination, which induce an immune response against stress proteins that is cross-protective against *M. tuberculosis*.

Immunogenic Constructs with HPV E7 as a Model Antigen

The present inventors and their colleagues previously developed several intracellular targeting and intercellular spreading strategies to enhance DNA vaccine potency using various IPP's (Hung, CF *et al., J Virol* 2002, 76:2676-2682; Cheng, WF *et al., J Clin Invest* 2001, 108:669-678;Hung, CF *et al., J Immunol* 2001, 166:5733-5740; Chen, CH *et al., Gene Ther* 1999, 6:1972-1981;Ji, H *et al., Hum Gene Ther* 1999, 10:2727-2740; Chen, CH *et al., Cancer Res* 2000, 60:1035-1042; U.S. Pat 6,734,173, WO 01/29233; WO03/085085; WO 02/012281;

WO 02/061113, *etc.*). Among these strategies, the linkage of *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) to human papillomavirus type 16 (HPV-16) E7 has been demonstrated to dramatically increase E7-specific $CD8^+$ T cell precursors and enhance anti-tumor effects aga1inst an E7-expressing tumor (TC-1) in vaccinated mice. These discoveries followed the earlier finding that immunization with HSP complexes isolated from tumor or virus-infected cells potentiated anti-tumor immunity (Janetzki, S *et al.,* 1998. *J Immunother 21*:269-76) or antiviral immunity (Heikema, AE *et al., Immunol Lett* 57:69-74). Immunogenic HSP-peptide complexes could be reconstituted *in vitro* by mixing the peptides with HSPs (Ciupitu, AM *et al.,* 1998. *J Exp Med* 187:685-91). Furthermore, HSP-based protein vaccines have been created by fusing antigens to HSPs (Suzue, K *et al.,* 1996. *J Immunol* 156:873-879). The results of these investigations point to HSPs one attractive candidate for use in immunotherapy. However, prior to the present inventors' work, HSP vaccines were peptide/protein-based vaccines.

Moreover, the present inventors and their colleagues were the first to provide naked DNA and self-replicating RNA vaccines that incorporated HSP70 and other immunogenicity-potentiating polypeptides. The present inventors and their colleagues also demonstrated that linking antigen to intracellular targeting moieties calreticulin (CRT), domain II of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)), or the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1) enhanced DNA vaccine potency compared to compositions comprising only DNA encoding the antigen of interest. To enhance MHC class II antigen processing, one of the present inventors and colleagues (Lin, KY *et al.*, 1996, *Canc Res 56*: 21-26) linked the sorting signals of the lysosome-associated membrane protein (LAMP-1) to the cytoplasmic/nuclear human papilloma virus (HPV-16) E7 antigen, creating a chimera (Sig/E7/LAMP-1). Expression of this chimera *in vitro* and *in vivo* with a recombinant vaccinia vector had targeted E7 to endosomal and lysosomal compartments and enhanced MHC class II presentation to CD4+ T cells. This vector was found to induce *in vivo* protection against an E7+ tumor, TC-1 so that 80% of mice vaccinated with the chimeric Sig/E7/LAMP1 vaccinia remained tumor free 3 months after tumor injection. Treatment with the Sig/E7/LAMP-1 vaccinia vaccine cured mice with small established TC-1 tumors, whereas the wild-type E7-vaccinia showed no effect on this established tumor burden. These findings point to the importance of adding an "element" to an antigenic composition to enhance *in vivo* potency of a

recombinant vaccine: in this case, a polypeptide that rerouted a cytosolic tumor antigen to the endosomal/lysosomal compartment

Intradermal administration of DNA vaccines via gene gun *in vivo* have proven to be an effective means to deliver such vaccines into professional antigen-presenting cells (APCs), primarily dendritic cells (DCs), which function in the uptake, processing, and presentation of antigen to T cells. The interaction between APCs and T cells is crucial for developing a potent specific immune response.

However, the various DNA or RNA constructs described by the present inventors or others in the prior art, have resulted in certain combinations that induced a heightened immune response in experimental animals. However, none of these vaccines have been ideally designed use in humans where administration may be limited for practical or other reasons to intramuscular injection. Because direct transduction of professional APCs in muscle tissue is not likely to occur due to paucity of such cells in muscle. That leaves cross-priming as the most likely mechanism for the induction of heightened immunity in humans. Optimizing vaccine constructs for cross priming requires that an element be added that promotes the secretion of the expressed polypeptide antigenic moiety, preferably as a fusion polypeptide with a molecule that promotes antigen processing via the MHC class I pathway. Moreover, it best to used plasmid constructs that are know to be safe and effective in humans. Finally, in the case of HPV oncoprotein antigens, it is also important to "detoxify" the protein that is to be expressed so that it will not act as an oncogenic transforming agent. It is to such constructs with the aforementioned advantageous properties that the present invention is directed.

SUMMARY OF THE INVENTION

The present inventors have designed and disclose herein an immunotherapeutic strategy that combines antigen-encoding DNA vaccine compositions which includes a signal peptide for secretion after initial uptake and expression, and a second protein, exemplified by HSP70, that promotes processing of the antigen via the MHC class I pathway and enhanced immunogenicity..

The growing understanding of the antigen presentation pathway creates the potential for designing novel strategies to enhance vaccine potency. One strategy taken by the present inventors in the present invention to enhance the presentation of antigen through the MHC class I pathway to $CD8^+$ T cells is the exploitation of the features of certain polypeptides to target antigenic polypeptide to which they are fused. Such polypeptide are referred to collectively

herein as "*i*mmunogenicity-potentiating (or -promoting) polypeptide" or "IPP" to reflect this general property, even though these IPP's may act by any of a number of cellular and molecular mechanisms that may or may not share common steps. This designation is intended to be interchangeable with the term "targeting polypeptide." Inclusion of nucleic acid sequences that encode polypeptides that modify the way the antigen encoded by molecular vaccine is "received" or "handled" by the immune system serve as a basis for enhancing vaccine potency.

The present invention is directed to a nucleic acid molecule encoding a fusion polypeptide useful as a vaccine composition, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide or peptide that promotes processing via the MHC class I pathway;

(b) fused in frame with the first nucleic acid sequence, a second nucleic acid sequence encoding a signal peptide; and

(c) a third nucleic acid sequence that is linked in frame to the first nucleic acid sequence and that encodes an antigenic polypeptide or peptide.

The antigenic peptide preferably comprises an epitope that binds to a MHC class I protein.

A preferred first polypeptide is Hsp70, an active C-terminal domain thereof, or a functional derivative of Hsp70 or of the C-terminal domain. Preferably this polypeptide is the *Mycobacterium tuberculosis* HSP70 and has the sequence SEQ ID NO:10 or is encoded by a nucleic acid SEQ ID NO:9.

The preferred antigenic polypeptide or peptide is one which is present on, or/ cross-reactive with an epitope of, a pathogenic organism, cell, or virus, preferably human papilloma virus. Preferred antigens are the E7 polypeptide of HPV-16 having the sequence SEQ ID NO:2, or an antigenic fragment thereof, or the E6 HPV polypeptide having the sequence SEQ ID NO:4. The third nucleic acid sequence of the above construct preferably encodes a non-oncogenic mutant or variant of the E7 or E6 protein, or both in tandem.

The preferred nucleic acid molecule above is pNGVL4a-Sig/E7(detox)/HSP70, and has the sequence SEQ ID NO:13.

Above the nucleic acid molecules may be linked to a promoter. of claim 1 operatively linked to a promoter.

The invention also includes an expression vector comprising any nucleic acid molecule as above, operatively linked to (a) a promoter; and (b) optionally, additional regulatory

sequences that regulate expression of the nucleic acid in a eukaryotic cell, preferably a human cell..

Also provided is a pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising the above nucleic acid molecule or expression vector and pharmaceutically and immunologically acceptable excipient.

Also included is a method of inducing or enhancing an antigen specific immune response in a subject, preferably a human, comprising administering to the subject an effective amount of the above pharmaceutical composition, thereby inducing or enhancing the response. The response is preferably one mediated at least in part by $CD8^+$ CTL.

The above method preferably comprises administering the pharmaceutical composition by intramuscular injection, by gene gun administration or by needle-free jet injection.

The invention is also directed to a method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to the subject an effective amount of the above pharmaceutical composition wherein the third nucleic acid sequence encodes one or more epitopes of E7 or E6, thereby inhibiting the growth or preventing the re-growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B show intracellular cytokine staining and flow cytometric analysis characterizing IFN-γ-secreting E7-specific $CD8^+$ T cell precursors in mice vaccinated with pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine via needle i.m., biojector, and gene gun. Flow cytometric analysis was of splenocytes collected from vaccinated mice one week after vaccination. and cultured *in vitro* with or without the E7 peptide (amino acids 49–57) overnight. Cells were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ secreting $CD8^+$ T cell precursors in naïve and immunized mice was analyzed. Fig. 1A shows representative results of intracellular cytokine staining. The number of $CD8^+$ IFN-γ double-positive T cells in $3x10^5$ splenocytes are indicated in the *upper right corner*. Fig. 1B shows composite result of flow cytometric analysis of IFN-γ-secreting E7-specific $CD8^+$ T cell precursors in naïve mice and mice vaccinated with pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine via needle i.m., biojector, and gene gun. Results are expressed as the mean number of number of E7-specific IFN-γ $CD8^+$ T lymphocytes per 3×10^5 splenocytes; *bars*, ± SE.

Figure 2 is a graph showing *in vivo* tumor protection experiments that compare the antitumor effects of the vaccine delivered by different routes. Mice were immunized and challenged with $5x10^4$ TC-1 tumor cells. Results are expressed as the percentage of tumor free mice at various days after tumor challenge.

Figures 3 and 4 are graphs showing results of *in vivo* tumor treatment experiments comparing antitumor effects of the vaccine delivered by different routes. Mice were inoculated i.v. with 10^4 TC-1 tumor cells via the tail vein and were subsequently treated with the DNA vaccine administered via needle i.m., by biojector. and by gene gun. Fig. 3 shows the number of pulmonary nodules present in naïve mice and in the groups of treated mice Results are expressed as the mean number of lung nodules; *bars*, ± SE. Fig. 4 shows weights of pulmonary nodules present in naïve mice and in groups of treated mice Results are expressed as the mean weight of lung nodules; *bars*, ± SE

Figure 5A is a schematic diagram of the pNGVL4a-Sig/E7(detox)/HSP70 plasmid vector used for anti-tumor vaccination. Indicated are various inserts and p

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Partial List of Abbreviations used

APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECD, extracellular domain; E6, HPV oncoprotein E6; E7, HPV oncoproteinE7; ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; HSP, heat shock protein; Hsp70, mycobacterial heat shock protein 70; IFNγ, interferon-γ; i.m., intramuscular(ly); i.v., intravenous(ly); MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; β-gal, β-galactosidase

The present inventors and their colleagues have shown that the linkage of *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) to human papillomavirus type 16 (HPV-16) oncoprotein E7 dramatically increased E7-specific $CD8^+$ T cell precursors and enhance anti-tumor effects against an E7-expressing tumor in vaccinated mice (Chen *et al.*, 2000, *supra*; US Pat. 6,734,173).

They have now adopted this strategy for Phase I/II clinical trials in patients with HPV-16 associated high-grade squamous intraepithelial lesion (HSIL) of the cervix and in patients with advanced HPV-associated head and neck squamous cell carcinoma (HNSCC). To do so, a GMP grade E7/HSP70 DNA vaccine was produced in the form of a naked DNA preparation based on the pNGVL4a plasmid (which plasmid was developed by the National Gene Vector Lab and has

been approved for use in humans). The DNA molecule of the present invention has been termed "pNGVL4a-Sig/E7(detox)/HSP70)". A similar DNA vaccine for the E6 protein is also described below.

The pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine was generated using the pNGVL4a vector as a backbone. This vector was originally derived from the pNGVL3 vector, which has been approved for human vaccine trials. The pNGVL4a vector includes two immunostimulatory sequences (tandem repeats of CpG dinucleotides) in the noncoding region. Whereas any other plasmid DNA that can transform either APCs or other cells which, via cross-priming, transfer the antigenic moiety to APCs, is useful in the present invention, PNGFVLA4 is preferred because of the fact that it has already been approved for human therapeutic use.

The pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine also comprises a signal peptide (Sig) the purpose of which is to enhance the immune response to DNA immunizations via the intramuscular (i.m.) route. The Sig acts by facilitating release of a chimeric E7/HSP70 polypeptide from cells which have taken up and expressed the chimeric polypeptide encoded by the DNA construct. In the case of i.m. immunization, due to the absence in muscle of a high density of professional antigen presenting cells (APC) and the fact that injection of the DNA would not directly target APCs, it is desired to induce immunity via cross-priming. If the immunogenic protein, such as the E7/HSP70 chimeric polypeptide, cannot leave the cell in which it is made, primarily myocytes after i.m. injection, cross-priming will not occur. The Sig sequence is exploited to permit and promote secretion of the E7/HSP70 from the muscle cells in which it is first expressed. Any signal sequence known to promote the sorting of an attached peptide into the rough endoplasmic reticulum, resulting in processing for secretion, may be used in place of the "Sig" sequence [residues encoded by nucleotides 4056-4127?? of SEQ ID NO:13. In the process of protein secretion, amino-terminal signal sequences are key recognition elements (see below). The limits of variation permitted for functional signal sequences can readily be determined by replacement of the normal signal sequence of a protein that is naturally targeted for secretion, or a reporter protein (*e.g.*, green fluorescent protein), with essentially random peptide sequences and testing for secretion. This has been done, for example, with yeast invertase by Kaiser CA *et al.*, *Science* 1987, 235:312-317.

As a protein begins to be assembled, the very first amino acids in the polypeptide chain indicate whether the protein is to be secreted or used in the cytosol. If a protein is destined for

secretion, the first amino acids are a special sequence called the signal sequence. The synthesis halts until the ribosome docks at the rough endoplasmic reticulum (RER). Once docked, the synthesis continues, with the new protein threading into the RER. The signal sequence is then cleaved from the polypeptide chain. Often, too, further enzymes cut the protein in other places. Most secreted proteins are modified before secretion. Next vesicles containing the protein bud from the RER and move to nearby the nearby Golgi apparatus. This is comprised of a stack of large, flattened vesicles. The vesicles from the RER fuse with one end, adding their proteins to the first flattened vesicle. In turn, small vesicles bud from this structure and transfer the protein to the next layer of the stack. This continues until the protein winds up at the opposite end of the Golgi apparatus. Further modification of the protein typically occurs here, for example, glycosylation (if the secreted protein is to be a glycoprotein). This renders the secreted protein more polar. Once the protein has moved through the entire Golgi apparatus, **secretion vesicles** containing the protein bud off, and move to and attach to the plasma membrane and release their contents into the extracellular fluid through the process of exocytosis.

The term “signal sequence” refers to a “signal peptide,” which is a peptide sequence that directs post translational uptake by organelles. Signal peptides are about 16-32 amino acids long are cleaved while proteins are still being processed. Signal peptides consist of one or more positively charged amino acids followed by 6-12 hydrophobic amino acids. Examples of such signal sequence may be found in any textbook of biochemistry or cell biology, for example, Albers, B. *et al.*, *Molecular Biology of the Cell*, 4th Ed., Garland Science, New York, NY (2002). One of ordinary skill in the art will readily appreciate how to select a signal sequence for use in accordance with this invention (as a substitute for the preferred “Sig” sequence noted herein).

I.M. immunization with a secreted form of an antigen, *i.e.*, that includes a signal peptide, will generate stronger CTL responses than i.m. immunization with a “cytoplasmic” form of antigen, suggesting that the priming of CTL responses after i.m. DNA immunization is facilitated by the cross-presentation of antigen by non-transfected professional APCs that have acquired the immunogen/antigen indirectly. See, for example, Boyle, JS *et al.*, *Int Immunol* 1997, 9:1897-1906.

In addition, when an oncoprotein or an epitope thereof is the immunizing moiety, it is necessary to reduce the tumorigenic risk of the vaccine itself. In the preferred embodiments, the

HPV E7 or E6 antigens are oncogenic. Thus the E7 protein was doubly mutated to a form known as "E7(detox) "by substituting two amino acids at positions $C^{24}G$ (Cys→Gly) and $E^{26}G$ (Glu→Gly). See SEQ ID NO:2. These substitutions completely eliminate the capacity of the E7 to binding capacity to Rb, as well as transforming activity.

Another embodiment of the present invention comprises an antigenic epitope of the HPV E6 protein, preferably from HPV-16. The E6 proteins from cervical cancer-associated HPV types such as HPV-16 induce proteolysis of the p53 tumor suppressor protein through interaction with E6-AP. Human mammary epithelial cells (MECs) immortalized by E6 display low levels of p53. HPV-16 E6 as well as other cancer-related papillomavirus E6 proteins also binds the cellular protein E6BP (ERC-55). Several different E6 mutations are discussed below after the "wild type" sequence of E6 is presented. The studies describing these mutants (which are incorporated by reference in their entirety) are also discussed in that section.

The present invention also includes the use of a tandem E6-E7 vaccine, using one or more of the mutations described herein to render the oncoproteins inactive with respect to their oncogenic potential *in vivo*. Cassetti MC *et al.*, Vaccine. 2004, 22:520-527, described Venezuelan equine encephalitis virus replicon particle (VRP) vaccines encoding the HPV16 E6 and E7 genes in which the E6 and E7 genes were fused to create one open reading frame and mutated at four or at five amino acid positions (see below). Thus, the present constructs may include one or more epitopes of E6 and E7, which may be arranged in their native order, resulting in either a E6-E7 or E7-E6 sequence, or shuffled in any way that permits the expressed protein to bear the E6 and E7 antigenic epitope(in an immunogenic form and result in immunization of the vaccinated recipient. DNA encoding amino acid spacers between E6 and E7 or between individual epitopes of these proteins may be introduced into the vector, provided again, that the spacers permit the expression or presentation of the epitopes in an immunogenic manner after they have been expressed by transduced host cells.

The order in which the two (or more) component polypeptides of the fusion protein are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can be altered without affecting immunogenicity of the fusion polypeptides proteins and the utility of the composition. For example, the Hsp70-encoding DNA sequences may be located 5' or 3' to the target antigen-encoding sequences. In one embodiment, these polypeptide-encoding nucleic acid domains are in-frame so that the DNA construct encodes a

recombinant fusion polypeptide in which the antigen is located N- terminal to the Hsp70 polypeptide. Of course, the signal peptide must be at the N-terminus of a nascent protein. Preferably, the DNA construct encodes a recombinant polypeptide in which the MHC class I restricted antigen, exemplified by mutant (detox) E7 (or E6) is located N-terminal to the HSP-derived residues.

Heat Shock Proteins and Homologues

Although the preferred DNA construct of the present invention encodes HSP70 from *M. tuberculosis*, any suitable heat shock protein (or stress protein) can be used in its place. Hsp60 and/or Hsp70 are preferred.

Families of stress genes and proteins that can be used in the present invention are those well known in the art and include, for example, Hsp100-200, Hsp100, Hsp90, Lon, Hsp70, Hsp60, TF55, Hsp40, FKBPs, cyclophilins, Hsp20-30, ClpP, GrpE, Hsp10, ubiquitin, calnexin, and protein disulfide isomerases (Macario, AJ Cold Spring Harbor Laboratory Res. 25:59-70, 1995; Parsell, DA *et al.*, *Ann. Rev. Genet. 27*:437-496 (1993); U.S. Pat. No. 5,232,833. A preferred group of stress proteins includes Hsp90, Hsp70, Hsp60, Hsp20-30; most preferred are Hsp70 and Hsp60.

Examples of Hsp100-200 include Grp170 (for glucose-regulated protein) which is found in the lumen of the ER, in the pre-Golgi compartment, and may play a role in immunoglobulin folding and assembly. Hsp100 members include mammalian Hsp110, yeast Hsp104, ClpA, ClpB, ClpC, ClpX and ClpY.

Examples of Hsp90 proteins include HtpG in *E. coli*, Hsp83 and Hsc83 yeast, and Hsp90α, Hsp90β and Grp94 in humans. Hsp90 binds cellular regulatory proteins such as steroid hormone receptors, transcription factors and protein kinases that play a role in signal transduction mechanisms. Hsp90 proteins also participate in the formation of large, abundant protein complexes that include other stress proteins.

Hsp70 examples include Hsp72 and Hsc73 from mammalian cells, products of the DnaK gene from bacteria, particularly mycobacteria such as *M. leprae, M. tuberculosis, and M. bovis* (such as BCG referred to herein as Hsp71), *E. coli*, other prokaryotes and yeast, and BiP and Grp78. Hsp70 can specifically binding ATP as well as unfolded polypeptides and peptides, thereby participating in protein folding and unfolding as well as in the assembly and disassembly of protein complexes.

Examples of Hsp60 proteins include Hsp65 from mycobacteria. Bacterial Hsp60 is also commonly known as GroEL, such as the GroEL from *E. coli*. Hsp60 forms large homo-oligomeric complexes, and appears to play a key role in protein folding. Hsp60 homologues are present in eukaryotic mitochondria and chloroplasts.

Examples of TF55 examples include Tcpl, TRiC and thermosome. The proteins typically occur in the cytoplasm of eukaryotes and some archaebacteria, and form multi-membered rings, promoting protein folding. They are also weakly homologous to Hsp60.

Examples of Hsp40 include DnaJ gene products from prokaryotes such as *E. coli* and mycobacteria and HSJ1, HDJ1 and Hsp40. Hsp40 plays a role as a molecular chaperone in protein folding, thermotolerance and DNA replication, among other cellular activities.

FKBPs examples include FKBP12, FKBP13, FKBP25, and FKBP59, Fprl and Nepl. The proteins typically have peptidyl-prolyl isomerase activity and interact with immunosuppressants such as FK506 and rapamycin. The proteins are typically found in the cytoplasm and the endoplasmic reticululum.

Cyclophilin examples include cyclophilins A, B and C. The proteins have peptidyl-prolyl isomerase activity and interact with the immunosuppressant cyclosporin A. The protein cyclosporin A binds calcineurin (a protein phosphatase).

Hsp20-30. also referred to as small Hsp, is typically found in large homo-oligomeric complexes or hetero-oligomeric complexes where an organism or cell expresses several different types of small Hsps. Hsp20-30 interacts with cytoskeletal structures, and may play a regulatory role in actin polymerization/depolymerization. Hsp20-30 is rapidly phosphorylated upon stressor exposure or exposure of resting cells to growth factors. Hsp20-30 homologues include α-crystallin.

ClpP is an *E. coli* protease involved in degradation of abnormal proteins. Homologues of ClpP are found in chloroplasts. ClpP forms a hetero-oligomeric complex with ClpA.

GrpE is an *E. coli* protein of about 20 kDa that is involved in both the rescue of stress-damaged proteins as well as the degradation of damaged proteins GrpE plays a role in the regulation of stress gene expression in *E. coli*.

Hsp10 examples include GroES and Cpn10. Hsp10 is typically found in *E. coli* and in mitochondria and chloroplasts of eukaryotic cells. Hsp10 forms a seven-membered ring that associates with Hsp60 oligomers. Hsp10 is also involved in protein folding.

Ubiquitin binds proteins in conjunction with proteolytic removal of the proteins by ATP-dependent cytosolic proteases.

In particular embodiments, the Hsp's of the present invention are derived from enterobacteria, mycobacteria (particularly *M. leprae, M. tuberculosis, M. vaccae, M. smegmatis and M. bovis), E. coli*, yeast, Drosophila, vertebrates, avians or mammals, including rats, mice and primates, including humans.

Homologues or variants of Hsp's as described herein, may also be used, provided that they have the requisite biological activity. These include various substitutions, deletions, or additions of the amino acid or nucleic acid sequences. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

The present invention may employ fragments of Hsp's provided such fragments can enhance the immune response to an antigen with which they are paired.

A preferred fragment is a C-terminal domain ("CD") of Hsp70, which is designated "$Hsp70_{CD}$". One $Hsp70_{CD}$ spans from about residue 312 to the C terminus of Hsp70 (SEQ ID NO:10). A preferred shorter polypeptide spans from about residue 517 to the C-terminus of SEQ ID NO:10. Shorter peptides from that sequence that have the ability to promote protein processing via the MHC-1 class I pathway are also included, and may be defined by routine experimentation.

A functional derivative of Hsp70 retains measurable Hsp70-like activity, preferably that of promoting immunogenicity of one or more antigenic epitopes fused thereto by promoting presentation by class I pathways. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous Hsp70 proteins including proteins not yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal

alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, Hsp70, SEQ ID NO:10). The amino acid residues (or nucleotides) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol. 48*:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-10. BLAST

nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to Hsp70 or FL nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of Hsp70 described above is characterized as having (a) functional activity of native Hsp70 and (b) sequence similarity to a native Hsp70 protein (such as SEQ ID NO:10) when determined as above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of Hsp70. Then, the fusion protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein, for example, a T cell proliferation, cytokine secretion or a cytolytic assay, or an *in vivo* assay of tumor protection or tumor therapy. A biological assay of the stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of Hsp70 refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A number of processes can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the spreading protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

A preferred group of variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of

protein chemistry and structure, see Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and their amides	Asp, Asn, Glu, Gln;
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

More substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of Gly and/or Pro by another amino acid or deletion or insertion of Gly or Pro; (ii) substitution of a hydrophilic residue, *e.g.*, Ser or Thr, for (or by) a hydrophobic residue, *e.g.*,, Leu, Ile, Phe, Val or Ala; (iii) substitution of a Cys residue for (or by) any other residue; (iv) substitution of a residue having an electropositive side chain, *e.g.*,, Lys, Arg or His, for (or by) a residue having an electronegative charge, *e.g.*,, Glu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.*, Phe, for (or by) a residue not having such a side chain, *e.g.*, Gly.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the wild-type or native

protein in terms of its intercellular spreading activity and its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the spreading protein. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

Whereas shorter chain variants can be made by chemical synthesis, for the present invention, the preferred longer chain variants are typically made by site-specific mutagenesis of the nucleic acid encoding the polypeptide, expression of the variant nucleic acid in cell culture, and, optionally, purification of the polypeptide from the cell culture, for example, by immunoaffinity chromatography using specific antibody immobilized to a column (to absorb the variant by binding to at least one epitope).

In the above composition the HSP70 or other stress protein preferably acts in potentiating an immune response by promoting: processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases the processing. This basic strategy may be combined with an additional strategy pioneered by the present inventors and colleagues, that involve linking DNA encoding another protein, generically termed a "targeting polypeptide, to the antigen-encoding DNA. Again, for the sake of simplicity, the DNA encoding such a targeting polypeptide will be referred to herein as a "targeting DNA." That strategy has been shown to be effective in enhancing the potency of the vectors carrying only antigen-encoding DNA. See for example: Wu *et al.*, WO 01/29233; Wu *et al.*, WO 02/009645; Wu *et al.*, WO 02/061113; Wu *et al.*, WO 02/074920; Wu *et al.*, WO 02/12281, all of which are incorporated by reference in their entirety. The other strategies include the use of DNA encoding polypeptides that promote or enhance:

(a) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of the antigen presenting cells leading to enhanced antigen presentation;

(b) intercellular transport and spreading of the antigen; or

(c) any combination of (a) and (b).

(d) sorting of the lysosome-associated membrane protein type 1 (Sig/LAMP-1)

(e) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus VP22 protein or a functional homologue or derivative thereof;

(f) an endoplasmic reticulum chaperone polypeptide selected from the group of calreticulin, ER60, GRP94, gp96, or a functional homologue or derivative thereof

(g) a cytoplasmic translocation polypeptide domains of a pathogen toxin selected from the group of domain II of *Pseudomonas* exotoxin ETA or a functional homologue or derivative thereof;

(h) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(i) a polypeptide that stimulates dendritic cell precursors or activates dendritic cell activity selected from the group of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof; or .

(j) a costimulatory signal, such as a B7 family protein, including B7-DC (see U.S. Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.*).

(k) an anti-apoptotic polypeptide preferably selected from the group consisting of (1) BCL-xL, (2) BCL2, (3) XIAP, (4) FLICEc-s, (5) dominant-negative caspase-8, (6) dominant negative caspase-9, (7) SPI-6, and (8) a functional homologue or derivative of any of (1)-(7).

The details of the various targeting polypeptide strategies will not be discussed in detail herein, although such vectors are used in the present examples, and their sequences are provided below. The preferred "targeting polypeptide" include the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1), which is the "Sig" in the preferred construct, the translocation domain (domain II or dII) of *Pseudomonas aeruginosa* exotoxin A (ETA(dII) (or from similar toxins from *Diphtheria*, *Clostridium*, *Botulinum*, *Bacillus*, *Yersinia*, *Vibrio cholerae*, or *Bordetella)*, an endoplasmic reticulum chaperone polypeptide exemplified by calreticulin (CRT) but also including ER60, GRP94 or gp96, well-characterized ER chaperone polypeptide that representatives of the HSP90 family of stress-induced proteins (see WO 02/012281), VP22 protein from herpes simplex virus and related herpes viruses such as Marek's disease virus (see WO 02/09645), and γ-tubulin. DNA encoding each of these polypeptides, or fragments or variants thereof with substantially the same biological activity, when linked to an antigen-encoding or epitope-encoding DNA molecule, and an HSP70 sequence result in more potent T cell mediate responses to the antigen compared to immunization with the antigen-encoding DNA alone or the antigen-HSP-70 chimeric construct. These polypeptide can be

considered as "molecular adjuvants." These effects are manifest primarily with CD8+ T cells, although some of these approaches induce potent CD4+ T cell mediated effects as well.

The experiments described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly HPV-16, are associated with most human cervical cancers. The oncogenic HPV proteins E7 and E6 are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 or E6 can be used to control of HPV-associated neoplasms (Wu (1994) *Curr. Opin. Immunol. 6*:746-754).

However, the present invention is not limited to the exemplified antigen(s). Rather, one of skill in the art will appreciate that the same results are expected for any antigen (and epitopes thereof) for which a T cell-mediated response is desired. The response so generated will be effective in providing protective or therapeutic immunity, or both, directed to an organism or disease in which the epitope or antigenic determinant is involved – for example as a cell surface antigen of a pathogenic cell or an envelope or other antigen of a pathogenic virus, or a bacterial antigen, or an antigen expressed as or as part of a pathogenic molecule.

Thus, in one embodiment, the antigen (*e.g.*, the MHC class I-binding peptide epitope) is derived from a pathogen, *e.g.*, it comprises a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g.*, a papilloma virus, a herpesvirus, a retrovirus (*e.g.*, an immunodeficiency virus, such as HIV-1), an adenovirus, and the like. The papilloma virus can be a human papilloma virus; for example, the antigen (*e.g.*, the Class I-binding peptide) can be derived from an HPV-16 E6 or E7 polypeptide. In a preferred embodiment, the HPV-16 E6 or E7 polypeptide used as an immunogen is substantially non-oncogenic, *i.e.*, it does not bind retinoblastoma polypeptide (pRB) or binds pRB with such low affinity that the HPV-16 E7 polypeptide is effectively non-oncogenic when expressed or delivered *in vivo*, which is accomplished as described herein.

In alternative embodiments, the pathogen is a bacteria, such as *Bordetella pertussis*; *Ehrlichia chaffeensis*; *Staphylococcus aureus*; *Toxoplasma gondii*; *Legionella pneumophila*; *Brucella suis*; *Salmonella enterica*; *Mycobacterium avium*; *Mycobacterium tuberculosis*;

Listeria monocytogenes; *Chlamydia trachomatis*; *Chlamydia pneumoniae*; *Rickettsia rickettsii*; or, a fungus, such as, *e.g.*, *Paracoccidioides brasiliensis*; or other pathogen, *e.g.*, *Plasmodium falciparum*.

In another embodiment, the MHC class I-binding antigenic peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, *e.g.*, a tumor specific antigen, such as, *e.g.*, a HER-2/neu antigen, or one of a number of known melanoma antigens, *etc.*.

In one embodiment, the isolated or recombinant nucleic acid molecule is operatively linked to a promoter, such as, *e.g.*, a constitutive, an inducible or a tissue-specific promoter. The promoter can be expressed in any cell, including cells of the immune system, including, *e.g.*, antigen presenting cells (APCs), *e.g.*, in a constitutive, an inducible or a tissue-specific manner.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered (or expressed *in vivo* by an administered nucleic acid, *e.g.*, a DNA vaccine) in an appropriate amount (an "immunogenically effective amount"), *i.e.*, capable of inducing, eliciting, augmenting or boosting a cellular and/or humoral immune response either alone or in combination or linked or fused to another substance (which can be administered at once or over several intervals). An immunogenic composition can comprise an antigenic peptide of at least about 5 amino acids, a peptide of 10 amino acids in length, a polypeptide fragment of 15 amino acids in length, 20 amino acids in length or longer. Smaller immunogens may require presence of a "carrier" polypeptide *e.g.*, as a fusion protein, aggregate, conjugate or mixture, preferably linked (chemically or otherwise) to the immunogen. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA comprising the immunogen's coding sequence operably linked to a promoter, *e.g.*, an expression cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids.

The term "vaccine" is used interchangeably with "immunogen" when referring to the DNA compositions of the present invention. Similarly, the terms "vaccinate" and "immunize" are used interchangeably here.

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.*, the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositions of the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or "epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class II protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro*, (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the HSP70 -encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself can be recombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

The term "self-replicating RNA replicon" refers to a construct based on an RNA viruses, such as alphavirus genome RNAs (*e.g.*, Sindbis virus, Semliki Forest virus, *etc.*), that have been engineered to allow expression of heterologous RNAs and proteins. These recombinant vectors are self-replicating ("replicons") which can be introduced into cells as naked RNA or DNA, as described in detail in co-pending, commonly assigned U.S. and PCT patent applications by several of the present inventors(U.S.S.N. 10/060,274, and WO 02/061113).

SEQUENCES OF POLYPEPTIDES AND NUCLEIC ACIDS

Plasmid and Vector Sequences

The wild-type HPV E7 sequence (nucleotide sequence is SEQ ID NO:1 used in the present invention, albeit with several mutations, and the wild-type amino acid sequence is SEQ

ID NO:2) is shown below. Underlined codons and amino acids are those which are preferably mutated in the present constructs.

```
1/1                                                 31/11
atg cat gga gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act
Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr
61/21                                   91/31
gat ctc tac tgt tat gag caa tta aat gac agc tca gag gag gag gat gaa ata gat ggt
Asp Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly
121/41                                  151/51
cca gct gga caa gca gaa ccg gac aga gcc cat tac aat att gta acc ttt tgt tgc aag
Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys
181/61                                  211/71
tgt gac tct acg ctt cgg ttg tgc gta caa agc aca cac gta gac att cgt act ttg gaa
Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu
241/81                                  271/91
gac ctg tta atg ggc aca cta gga att gtg tgc ccc atc tgt tct cag gat aag ctt
Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln Asp Lys Leu
```

The above sequence differs from the GENEBANKAccession Number NC_001526 for the E7 protein which is:

MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK
CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP 97 (SEQ ID NO:3)

The HPV16 E7 protein binds Rb through an L–X–C–X–E motif. Mutations at positions Cys24 and Glu26 of this motif destroy Rb binding and degradation. In addition to these two point mutations in E7, a mutation at a third amino acid, Cys91, destroys the single zinc finger in E7. In a preferred embodiment, all wild type amino acids are mutated to Gly. In another embodiment, these residues are mutated to Ala. In fact, they can be mutated to any residue that will permit the protein to be expressed in transduced cells, secreted in immunogenic form, taken up by professional APCs, and presented to T cells in a way that will preserve antigenic specificity, while at the same time preventing or lowering the probability that the protein will have oncogenic transforming capacity. The above statement is true with respect to the HPV E6 protein described below.

To reduce oncogenic potential of E7 in a construct of this invention, one or more of the following positions of E7 is mutated:

Original residue	Mutant residue	Preferred codon mutation	Position in SEQ ID NO:2
Cys	Gly (or Ala)	TGT→ GGT	24
Glu	Gly (or Ala)	GAG→ GGG (or GCG)	26
Cys	Gly (or Ala)	TGC→ GGC	91

The E7 (detox) mutant sequence included in the preferred vaccine vector (SEQ ID NO:13) has the mutations shown in Figure 5B- namely - a tgt→ ggt mutation resulting in a

Cys→ Gly substitution at position 24 of SEQ ID NO:2 and a gag→ ggg mutation resulting in a Glu→ Gly substitution at position 26 or SEQ ID NO:2.

E6 Protein

The wild type HPV E6 amino acid sequence (GENEBANK Accession Number NC_001526) (SEQ ID NO:4) is shown below. This sequence has 158 amino acids.

MHQKRTAMFQ DPQERPRKLP QLCTELQTTI HDIILECVYC KQQLLRREVY DFAFRDLCIV
YRDGNPYAVC DKCLKFYSKI SEYRHYCYSL YGTTLEQQYN KPLCDLLIRC INCQKPLCPE
EKQRHLDKKQ RFHNIRGRWT GRCMSCCRSS RTRRETQL

The preferred amino acid residues to be mutated (as discussed below) are underscored above. The studies of E6 mutants discussed below are based upon a different E6 sequence, of 151 nucleic acids, wherein the N-terminal residue was considered to be the Met at position 8 in SEQ ID NO:4. That shorter version of E6 is shown below as SEQ ID NO:5.

MFQDPQERPR KLPQLCTELQ TTIHDIILEC VYCKQQLLRR EVYDFAFRDL CIVYRDGNPY
AVCDKCLKFY SKISEYRHYC YSLYGTTLEQ QYNKPLCDLL IRCINCQKPL CPEEKQRHLD
KKQRFHNIRG RWTGRCMSCC RSSRTRRETQ L

The preferred amino acid residues to be mutated (as discussed below) are underscored above.

Any nucleotide sequence that encodes encoding this E6 polypeptide, or preferably, one of the mutants thereof discussed below, or an antigenic fragment or epitope thereof, can be used in the present vectors. Other mutations can be tested and used in accordance with the methods described herein, or those described by Cassatti *et al.*, *supra*.

To reduce oncogenic potential of E6 in a construct of this invention, one or more of the following positions of E6 is mutated:

Original residue	Mutant residue	Position in SEQ ID NO:4	Position in SEQ ID NO:5
Ile	Thr	135	128
Cys	Gly (or Ala)	70	63
Cys	Gly (or Ala)	113	106

These mutations can be achieved using any appropriate coding sequences by mutation of the coding DNA.

The studies describing these mutants (which are incorporated by reference in their entirety) are discussed below. Nguyen M *et al., J Virol.* 2002, 6:13039-13048, described a mutant of HPV-16 E6 deficient in binding α-helix partners which displays reduced oncogenic potential *in vivo*. This mutant, that involves a replacement of Ile with Thr as position 128 (of SEQ ID NO:5) , may be used in accordance with the present invention to make an E6 DNA vaccine that has a lower risk of being oncogenic. This E6(I^{128}T) mutant is defective for binding at least a subset of the α-helix partners, including E6AP, the ubiquitin ligase that mediates E6-dependent degradation of the p53 protein,

Cassetti *et al.*, *supra*, examined the effects of mutations four or five amino acid positions in E6 and E7 to inactivate their oncogenic potential. The following mutations were examined (positions based on SEQ ID NO:5): E6-C^{63}G; E6 C^{106}G; E7-C^{24}G, E7-E^{26}G, and E7 C^{91}G. Vaccines encoding mutant or wild type E6 and E7 proteins elicited comparable CTL responses and generated comparable antitumor responses in several HPV16 E6(+)E7(+) tumor challenge models: protection from either C3 or TC-1 tumor challenge was observed in 100% of vaccinated mice. Eradication of C3 tumors was observed in approximately 90%. The predicted inactivation of E6 and E7 oncogenic potential was confirmed by demonstrating normal levels of both p53 and Rb proteins in human mammary epithelial cells infected with VRP expressing mutant E6 and E7 genes.

Approaches for Mutagenesis of E6 and E7

The HPV16 E6 protein contains two zinc fingers important for structure and function; one cysteine (C) amino acid position in each pair of C–X–X–C (where X is any amino acid) zinc finger motifs are preferably was mutated at E6 positions 63 and 106 (based on SEQ ID NO:5). Mutants are created, for example, using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HPV16 E6 containing a single point mutation at Cys106 (of Cys 113 per SEQ ID NO:4). Cys neither binds nor facilitates degradation of p53 and is incapable of immortalizing human mammary epithelial cells (MEC), a phenotype dependent upon p53 degradation. A single amino acid substitution at position Cys63 of SEQ ID NO:3 (=Cys70 in SEQ ID NO:4) destroys several HPV16 E6 functions: p53 degradation, E6TP-1 degradation, activation of telomerase, and, consequently, immortalization of primary epithelial cells.

Sequences of DNA Encoding Immunogenicity-Potentiating Polypeptides and their Vectors

The preferred nucleotide [SEQ ID NO:6] and amino acid sequence [SEQ ID NO:7] of Sig/E7 are as follows: the Sig sequence is underscored:

```
MAAPGARRPL LLLLLAGLAH GASALFEDLI MHGDTPTLHE YMLDLQPETT DLYCYEQLND
SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV
CPICSQP
```

A preferred nucleic acid sequence encoding a 30 residue Sig sequence is shown below (SEQ ID NO:8).

```
atg gcg gcc ccc ggc gcc cgg cgg ccg ctg ctc ctg ctg ctg ctg
gca ggc ctt gca cat ggc gcc tca gca ctc ttt gag gat cta atc
```

In another embodiment, the sequence is a C-terminally truncated version of SEQ ID NO:8, encoding from about 15-25 of the N-terminal residues of SEQ ID NO:8. As noted elsewhere, any known signal sequence may be substituted for this one in the present construct.

<u>HSP70 from *M. tuberculosis*</u>

The nucleotide sequence encoding HSP70 (SEQ ID NO:9) is shown below and is deposited in GENBANK; nucleotides 10633-12510 of the *M. tuberculosis* genome.

```
 atggctcg tgcggtcggg atcgacctcg ggaccaccaa ctccgtcgtc tcggttctgg aaggtggcga
cccggtcgtc gtcgccaact ccgagggctc caggaccacc ccgtcaattg tcgcgttcgc ccgcaacggt
gaggtgctgg tcggccagcc cgccaagaac caggcagtga ccaacgtcga tcgcaccgtg cgctcggtca
agcgacacat gggcagcgac tggtccatag agattgacgg caagaaatac accgcgccgg agatcagcgc
ccgcattctg atgaagctga agcgcgacgc cgaggcctac ctcggtgagg acattaccga cgcggttatc
acgacgcccg cctacttcaa tgacgcccag cgtcaggcca ccaaggacgc cggccagatc gccggcctca
acgtgctgcg gatcgtcaac gagccgaccg cggccgcgct ggcctacggc ctcgacaagg gcgagaagga
gcagcgaatc ctggtcttcg acttgggtgg tggcactttc gacgtttccc tgctggagat cggcgagggt
gtggttgagg tccgtgccac ttcgggtgac aaccacctcg gcggcgacga ctgggaccag cgggtcgtcg
attggctggt ggacaagttc aagggcacca gcggcatcga tctgaccaag gacaagatgg cgatgcagcg
gctgcgggaa gccgccgaga aggcaaagat cgagctgagt tcgagtcagt ccacctcgat caacctgccc
tacatcaccg tcgacgccga caagaacccg ttgttcttag acgagcagct gacccgcgcg gagttccaac
ggatcactca ggacctgctg gaccgcactc gcaagccgtt ccagtcggtg atcgctgaca ccggcatttc
ggtgtcggag atcgatcacg ttgtgctcgt gggtggttcg acccggatgc ccgcggtgac cgatctggtc
aaggaactca ccggcggcaa ggaacccaac aagggcgtca accccgatga ggttgtcgcg gtgggagccg
ctctgcaggc cggcgtcctc aagggcgagg tgaaagacgt tctgctgctt gatgttaccc cgctgagcct
gggtatcgag accaagggcg ggtgatgac caggctcatc gagcgcaaca ccacgatccc caccaagcgg
tcggagactt tcaccaccgc cgacgacaac caaccgtcgg tgcagatcca ggtctatcag gggagcgtg
agatcgccgc gcacaacaag ttgctcgggt ccttcgagct gaccggcatc ccgccggcgc cgcgggggat
tccgcagatc gaggtcactt tcgacatcga cgccaacggc attgtgcacg tcaccgccaa ggacaagggc
accggcaagg agaacacgat ccgaatccag gaaggctcgg gcctgtccaa ggaagacatt gaccgcatga
tcaaggacgc cgaagcgcac gccgaggagg atcgcaagcg tcgcgaggag gccgatgttc gtaatcaagc
cgagacattg gtctaccaga cggagaagtt cgtcaaagaa cagcgtgagg ccgagggtgg ttcgaaggta
cctgaagaca cgctgaacaa ggttgatgcc gcggtggcgg aagcgaaggc ggcacttggc ggatcggata
tttcggccat caagtcggcg atggagaagc tgggccagga gtcgcaggct ctggggcaag cgatctacga
agcagctcag gctgcgtcac aggccactgg cgctgcccac cccggcggcg agccgggcgg tgcccacccc
ggctcggctg atgacgttgt ggacgcggag gtggtcgacg acggccggga ggccaagtga
```

The amino acid sequence of HSP70 [SEQ ID NO:10] is:

```
MARAVGIDLG TTNSVVSVLE GGDPVVVANS EGSRTTPSIV AFARNGEVLV GQPAKNQAVT NVDRTVRSVK
RHMGSDWSIE IDGKKYTAPE ISARILMKLK RDAEAYLGED ITDAVITTPA YFNDAQRQAT KDAGQIAGLN
VLRIVNEPTA AALAYGLDKG EKEQRILVFD LGGGTFDVSL LEIGEGVVEV RATSGDNHLG GDDWDQRVVD
WLVDKFKGTS GIDLTKDKMA MQRLREAAEK AKIELSSSQS TSINLPYITV DADKNPLFLD EQLTRAEFQR
ITQDLLDRTR KPFQSVIADT GISVSEIDHV VLVGGSTRMP AVTDLVKELT GGKEPNKGVN PDEVVAVGAA
LQAGVLKGEV KDVLLLDVTP LSLGIETKGG VMTRLIERNT TIPTKRSETF TTADDNQPSV QIQVYQGERE
```

```
IAAHNKLLGS FELTGIPPAP RGIPQIEVTF DIDANGIVHV TAKDKGTGKE NTIRIQEGSG LSKEDIDRMI
KDAEAHAEED RKRREEADVR NQAETLVYQT EKFVKEQREA EGGSKVPEDT LNKVDAAVAE AKAALGGSDI
SAIKSAMEKL GQESQALGQA IYEAAQAASQ ATGAAHPGGE PGGAHPGSAD DVVDAEVVDD GREAK    625
```

A preferred E7-Hsp70 chimera or fusion (nucleic acid is SEQ ID NO:11; amino acids are SEQ ID NO:12) is shown below (using the wild-type E7 coding sequence which is capitalized, is shown below. For the present construct, the relevant codons/residues discussed above are mutated as described..

```
1/1                                     31/11
ATG CAT GGA GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT
Met his gly asp thr pro thr leu his glu tyr met leu asp leu gln pro glu thr thr

61/21                                   91/31
GAT CTC TAC TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT
asp leu tyr cys tyr glu gln leu asn asp ser ser glu glu glu asp glu ile asp gly
121/41                                  151/51
CCA GCT GGA CAA GCA GAA CCG GAC AGA GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG
pro ala gly gln ala glu pro asp arg ala his tyr asn ile val thr phe cys cys lys
181/61                                  211/71
TGT GAC TCT ACG CTT CGG TTG TGC GTA CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA
cys asp ser thr leu arg leu cys val gln ser thr his val asp ile arg thr leu glu
241/81                                  271/91
GAC CTG TTA ATG GGC ACA CTA GGA ATT GTG TGC CCC ATC TGT TCT CAA GGA TCC atg gct
asp leu leu met gly thr leu gly ile val cys pro ile cys ser gln gly ser met ala
301/101                                 331/111
cgt gcg gtc ggg atc gac ctc ggg acc acc aac tcc gtc gtc tcg gtt ctg gaa ggt ggc
arg ala val gly ile asp leu gly thr thr asn ser val val ser val leu glu gly gly
361/121                                 391/131
gac ccg gtc gtc gtc gcc aac tcc gag ggc tcc agg acc acc ccg tca att gtc gcg ttc
asp pro val val val ala asn ser glu gly ser arg thr thr pro ser ile val ala phe
421/141                                 451/151
gcc cgc aac ggt gag gtg ctg gtc ggc cag ccc gcc aag aac cag gca gtg acc aac gtc
ala arg asn gly glu val leu val gly gln pro ala lys asn gln ala val thr asn val
481/161                                 511/171
gat cgc acc gtg cgc tcg gtc aag cga cac atg ggc agc gac tgg tcc ata gag att gac
asp arg thr val arg ser val lys arg his met gly ser asp trp ser ile glu ile asp
541/181                                 571/191
ggc aag aaa tac acc gcg ccg gag atc agc gcc cgc att ctg atg aag ctg aag cgc gac
gly lys lys tyr thr ala pro glu ile ser ala arg ile leu met lys leu lys arg asp
601/201                                 631/211
gcc gag gcc tac ctc ggt gag gac att acc gac gcg gtt atc acg acg ccc gcc tac ttc
ala glu ala tyr leu gly glu asp ile thr asp ala val ile thr thr pro ala tyr phe
661/221                                 691/231
aat gac gcc cag cgt cag gcc acc aag gac gcc ggc cag atc gcc ggc ctc aac gtg ctg
asn asp ala gln arg gln ala thr lys asp ala gly gln ile ala gly leu asn val leu
721/241                                 751/251
cgg atc gtc aac gag ccg acc gcg gcc gcg ctg gcc tac ggc ctc gac aag ggc gag aag
arg ile val asn glu pro thr ala ala ala leu ala tyr gly leu asp lys gly glu lys
781/261                                 811/271
gag cag cga atc ctg gtc ttc gac ttg ggt ggt ggc act ttc gac gtt tcc ctg ctg gag
glu gln arg ile leu val phe asp leu gly gly gly thr phe asp val ser leu leu glu
841/281                                 871/291
atc ggc gag ggt gtg gtt gag gtc cgt gcc act tcg ggt gac aac cac ctc ggc ggc gac
ile gly glu gly val val glu val arg ala thr ser gly asp asn his leu gly gly asp
901/301                                 931/311
gac tgg gac cag cgg gtc gtc gat tgg ctg gtg gac aag ttc aag ggc acc agc ggc atc
asp trp asp gln arg val val asp trp leu val asp lys phe lys gly thr ser gly ile
961/321                                 991/331
gat ctg acc aag gac aag atg gcg atg cag cgg ctg cgg gaa gcc gcc gag aag gca aag
asp leu thr lys asp lys met ala met gln arg leu arg glu ala ala glu lys ala lys
1021/341                                1051/351
atc gag ctg agt tcg agt cag tcc acc tcg atc aac ctg ccc tac atc acc gtc gac gcc
ile glu leu ser ser ser gln ser thr ser ile asn leu pro tyr ile thr val asp ala
1081/361                                1111/371
gac aag aac ccg ttg ttc tta gac gag cag ctg acc cgc gcg gag ttc caa cgg atc act
asp lys asn pro leu phe leu asp glu gln leu thr arg ala glu phe gln arg ile thr
1141/381                                1171/391
```

```
cag gac ctg ctg gac cgc act cgc aag ccg ttc cag tcg gtg atc gct gac acc ggc att
gln asp leu leu asp arg thr arg lys pro phe gln ser val ile ala asp thr gly ile
1201/401                                1231/411
tcg gtg tcg gag atc gat cac gtt gtg ctc gtg ggt ggt tcg acc cgg atg ccc gcg gtg
ser val ser glu ile asp his val val leu val gly gly ser thr arg met pro ala val
1261/421                                1291/431
acc gat ctg gtc aag gaa ctc acc ggc ggc aag gaa ccc aac aag ggc gtc aac ccc gat
thr asp leu val lys glu leu thr gly gly lys glu pro asn lys gly val asn pro asp
1321/441                                1351/451
gag gtt gtc gcg gtg gga gcc gct ctg cag gcc ggc gtc ctc aag ggc gag gtg aaa gac
glu val val ala val gly ala ala leu gln ala gly val leu lys gly glu val lys asp
1381/461                                1411/471
gtt ctg ctg ctt gat gtt acc ccg ctg agc ctg ggt atc gag acc aag ggc ggg gtg atg
val leu leu leu asp val thr pro leu ser leu gly ile glu thr lys gly gly val met
1441/481                                1471/491
acc agg ctc atc gag cgc aac acc acg atc ccc acc aag cgg tcg gag act ttc acc acc
thr arg leu ile glu arg asn thr thr ile pro thr lys arg ser glu thr phe thr thr
1501/501                                1531/511
gcc gac gac aac caa ccg tcg gtg cag atc cag gtc tat cag ggg gag cgt gag atc gcc
ala asp asp asn gln pro ser val gln ile gln val tyr gln gly glu arg glu ile ala
1561/521                                1591/531
gcg cac aac aag ttg ctc ggg tcc ttc gag ctg acc ggc atc ccg ccg gcg ccg cgg ggg
ala his asn lys leu leu gly ser phe glu leu thr gly ile pro pro ala pro arg gly
1621/541                                1651/551
att ccg cag atc gag gtc act ttc gac atc gac gcc aac ggc att gtg cac gtc acc gcc
ile pro gln ile glu val thr phe asp ile asp ala asn gly ile val his val thr ala
1681/561                                1711/571
aag gac aag ggc acc ggc aag gag aac acg atc cga atc cag gaa ggc tcg ggc ctg tcc
lys asp lys gly thr gly lys glu asn thr ile arg ile gln glu gly ser gly leu ser
1741/581                                1771/591
aag gaa gac att gac cgc atg atc aag gac gcc gaa gcg cac gcc gag gag gat cgc aag
lys glu asp ile asp arg met ile lys asp ala glu ala his ala glu glu asp arg lys
1801/601                                1831/611
cgt cgc gag gag gcc gat gtt cgt aat caa gcc gag aca ttg gtc tac cag acg gag aag
arg arg glu glu ala asp val arg asn gln ala glu thr leu val tyr gln thr glu lys
1861/621                                1891/631
ttc gtc aaa gaa cag cgt gag gcc gag ggt ggt tcg aag gta cct gaa gac acg ctg aac
phe val lys glu gln arg glu ala glu gly gly ser lys val pro glu asp thr leu asn
1921/641                                1951/651

aag gtt gat gcc gcg gtg gcg gaa gcg aag gcg gca ctt ggc gga tcg gat att tcg gcc
lys val asp ala ala val ala glu ala lys ala ala leu gly gly ser asp ile ser ala
1981/661                                2011/671
atc aag tcg gcg atg gag aag ctg ggc cag gag tcg cag gct ctg ggg caa gcg atc tac
ile lys ser ala met glu lys leu gly gln glu ser gln ala leu gly gln ala ile tyr
2041/681                                2071/691
gaa gca gct cag gct gcg tca cag gcc act ggc gct gcc cac ccc ggc tcg gct gat gaA
glu ala ala gln ala ala ser gln ala thr gly ala ala his pro gly ser ala asp glu
2101/701
AGC a
ser
```

A preferred nucleotide sequence for the entire DNA vaccine vector is shown below (SEQ ID NO:13

1 GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT

51 GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC

101 TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC

151 CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA

201 GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC

251 GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT

301 TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG

351 GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG

401 AAGTGGTGGC CTAACTACGG CTACACTAGA AGAACAGTAT TTGGTATCTG

451 CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT

501 CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

551 CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC

601 TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG

651 TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA

701 TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG

751 TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC

801 GTTCATCCAT AGTTGCCTGA CTCGGGGGGG GGGGGCGCTG AGGTCTGCCT

851 CGTGAAGAAG GTGTTGCTGA CTCATACCAG GGCAACGTTG TTGCCATTGC

901 TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT

951 CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA

1001 AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC

1051 CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG

1101 TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG

1151 TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC

1201 AATACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA
1251 TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG
1301 AGATCCAGTT CGATGTAACC CACTCGTGCA CCTGAATCGC CCCATCATCC
1351 AGCCAGAAAG TGAGGGAGCC ACGGTTGATG AGAGCTTTGT TGTAGGTGGA
1401 CCAGTTGGTG ATTTTGAACT TTTGCTTTGC CACGGAACGG TCTGCGTTGT
1451 CGGGAAGATG CGTGATCTGA TCCTTCAACT CAGCAAAAGT TCGATTTATT
1501 CAACAAAGCC GCCGTCCCGT CAAGTCAGCG TAATGCTCTG CCAGTGTTAC
1551 AACCAATTAA CCAATTCTGA TTAGAAAAAC TCATCGAGCA TCAAATGAAA
1601 CTGCAATTTA TTCATATCAG GATTATCAAT ACCATATTTT TGAAAAAGCC
1651 GTTTCTGTAA TGAAGGAGAA AACTCACCGA GGCAGTTCCA TAGGATGGCA
1701 AGATCCTGGT ATCGGTCTGC GATTCCGACT CGTCCAACAT CAATACAACC
1751 TATTAATTTC CCCTCGTCAA AAATAAGGTT ATCAAGTGAG AAATCACCAT
1801 GAGTGACGAC TGAATCCGGT GAGAATGGCA AAAGCTTATG CATTTCTTTC
1851 CAGACTTGTT CAACAGGCCA GCCATTACGC TCGTCATCAA AATCACTCGC
1901 ATCAACCAAA CCGTTATTCA TTCGTGATTG CGCCTGAGCG AGACGAAATA
1951 CGCGATCGCT GTTAAAAGGA CAATTACAAA CAGGAATCGA ATGCAACCGG
2001 CGCAGGAACA CTGCCAGCGC ATCAACAATA TTTTCACCTG AATCAGGATA
2051 TTCTTCTAAT ACCTGGAATG CTGTTTTCCC GGGGATCGCA GTGGTGAGTA
2101 ACCATGCATC ATCAGGAGTA CGGATAAAAT GCTTGATGGT CGGAAGAGGC
2151 ATAAATTCCG TCAGCCAGTT TAGTCTGACC ATCTCATCTG TAACATCATT
2201 GGCAACGCTA CCTTTGCCAT GTTTCAGAAA CAACTCTGGC GCATCGGGCT
2251 TCCCATACAA TCGATAGATT GTCGCACCTG ATTGCCCGAC ATTATCGCGA
2301 GCCCATTTAT ACCCATATAA ATCAGCATCC ATGTTGGAAT TTAATCGCGG
2351 CCTCGAGCAA GACGTTTCCC GTTGAATATG GCTCATAACA CCCCTTGTAT
2401 TACTGTTTAT GTAAGCAGAC AGTTTTATTG TTCATGATGA TATATTTTTA
2451 TCTTGTGCAA TGTAACATCA GAGATTTTGA GACACAACGT GGCTTTCCCC
2501 CCCCCCCCAT TATTGAAGCA TTTATCAGGG TTATTGTCTC ATGAGCGGAT
2551 ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA

2601 TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA TTATCATGAC
2651 ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTCGCGCGTT
2701 TCGGTGATGA CGGTGAAAAC CTCTGACACA TGCAGCTCCC GGAGACGGTC
2751 ACAGCTTGTC TGTAAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC
2801 GTCAGCGGGT GTTGGCGGGT GTCGGGGCTG GCTTAACTAT GCGGCATCAG
2851 AGCAGATTGT ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA
2901 TGCGTAAGGA GAAAATACCG CATCAGATTG GCTATTGGCC ATTGCATACG
2951 TTGTATCCAT ATCATAATAT GTACATTTAT ATTGGCTCAT GTCCAACATT
3001 ACCGCCATGT TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA
3051 CGGGGTCATT AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT
3101 ACGGTAAATG GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC
3151 GTCAATAATG ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT
3201 GACGTCAATG GGTGGAGTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT
3251 CAAGTGTATC ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA
3301 ATGGCCCGCC TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA
3351 CTTGGCAGTA CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG
3401 TTTTGGCAGT ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT
3451 TCCAAGTCTC CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA
3501 ATCAACGGGA CTTTCCAAAA TGTCGTAACA ACTCCGCCCC ATTGACGCAA
3551 ATGGGCGGTA GGCGTGTACG GTGGGAGGTC TATATAAGCA GAGCTCGTTT
3601 AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC
3651 ATAGAAGACA CCGGGACCGA TCCAGCCTCC GCGGCCGGGA ACGGTGCATT
3701 GGAACGCGGA TTCCCCGTGC CAAGAGTGAC GTAAGTACCG CCTATAGACT
3751 CTATAGGCAC ACCCCTTTGG CTCTTATGCA TGCTATACTG TTTTTGGCTT
3801 GGGGCCTATA CACCCCCGCT TCCTTATGCT ATAGGTGATG GTATAGCTTA
3851 GCCTATAGGT GTGGGTTATT GACCATTATT GACCACTCCA ACGGTGGAGG
3901 GCAGTGTAGT CTGAGCAGTA CTCGTTGCTG CCGCGCGCGC CACCAGACAT
3951 AATAGCTGAC AGACTAACAG ACTGTTCCTT TCCATGGGTC TTTTCTGCAG
4001 TCACCGTCGT CGACGGTATC GATAAGCTTG ATATCGAATT CCTCGACGGA

4051 TCTTATGGCG GCCCCCGGCG CCCGGCGGCC GCTGCTCCTG CTGCTGCTGG
4101 CAGGCCTTGC AGATGGCGCC TCAGCACTCT TTGAGGATCT AATCATGCAT
4151 GGAGATACAC CTACATTGCA TGAATATATG TTAGATTTGC AACCAGAGAC
4201 AACTGATCTC TACGGTTATG GGCAATTAAA TGACAGCTCA GAGGAGGAGG
4251 ATGAAATAGA TGGTCCAGCT GGACAAGCAG AACCGGACAG AGCCCATTAC
4301 AATATTGTAA CCTTTTGTTG CAAGTGTGAC TCTACGCTTC GGTTGTGCGT
4351 ACAAAGCACA CACGTAGACA TTCGTACTTT GGAAGACCTG TTAATGGGCA
4401 CACTAGGAAT TGTGTGCCCC ATCTGTTCTC AAGGATCCAT GGCTCGTGCG
4451 GTCGGGATCG AGCTCGGGAC CACCAACTCC GTCGTCTCGG TTCTGGAAGG
4501 TGGCGACCCG GTCGTCGTCG CCAACTCCGA GGGCTCCAGG ACCACCCCGT
4551 CAATTGTCGC GTTCGCCCGC AACGGTGAGG TGCTGGTCGG CCAGCCCGCC
4601 AAGAACCAGG CGGTGACCAA CGTCGATCGC ACCGTGCGCT CGGTCAAGCG
4651 ACACATGGGC AGCGACTGGT CCATAGAGAT TGACGGCAAG AAATACACCG
4701 CGCCGGAGAT CAGCGCCCGC ATTCTGATGA AGCTGAAGCG CGACGCCGAG
4751 GCCTACCTCG GTGAGGACAT TACCGACGCG GTTATCACGA CGCCCGCCTA
4801 CTTCAATGAC GCCCAGCGTC AGGCCACCAA GGACGCCGGC CAGATCGCCG
4851 GCCTCAACGT GCTGCGGATC GTCAACGAGC CGACCGCGGC CGCGCTGGCC
4901 TACGGCCTCG ACAAGGGCGA GAAGGAGCAG CGAATCCTGG TCTTCGACTT
4951 GGGTGGTGGC ACTTTCGACG TTTCCCTGCT GGAGATCGGC GAGGGTGTGG
5001 TTGAGGTCCG TGCCACTTCG GGTGACAACC ACCTCGGCGG CGACGACTGG
5051 GACCAGCGGG TCGTCGATTG GCTGGTGGAC AAGTTCAAGG GCACCAGCGG
5101 CATCGATCTG ACCAAGGACA AGATGGCGAT GCAGCGGCTG CGGGAAGCCG
5151 CCGAGAAGGC AAAGATCGAG CTGAGTTCGA GTCAGTCCAC CTCGATCAAC
5201 CTGCCCTACA TCACCGTCGA CGCCGACAAG AACCCGTTGT TCTTAGACGA
5251 GCAGCTGACC CGCGCGGAGT TCCAACGGAT CACTCAGGAC CTGCTGGACC
5301 GCACTCGCAA GCCGTTCCAG TCGGTGATCG CTGACACCGG CATTTCGGTG
5351 TCGGAGATCG ATCACGTTGT GCTCGTGGGT GGTTCGACCC GGATGCCCGC
5401 GGTGACCGAT CTGGTCAAGG AACTCACCGG CGGCAAGGAA CCCAACAAGG

5451 GCGTCAACCC CGATGAGGTT GTCGCGGTGG GAGCCGCTCT GCAGGCCGGC
5501 GTCCTCAAGG GCGAGGTGAA AGACGTTCTG CTGCTTGATG TTACCCCGCT
5551 GAGCCTGGGT ATCGAGACCA AGGGCGGGGT GATGACCAGG CTCATCGAGC
5601 GCAACACCAC CATCCCCACC AAGCGGTCGG AGACTTTCAC CACCGCCGAC
5651 GACAACCAAC CGTCGGTGCA GATCCAGGTC TATCAGGGGG AGCGTGAGAT
5701 CGCCGCGCAC AACAAGTTGC TCGGGTCCTT CGAGCTGACC GGCATCCCGC
5751 CGGCGCCGCG GGGGATTCCG CAGATCGAGG TCACTTTCGA CATCGACGCC
5801 AACGGCATTG TGCACGTCAC CGCCAAGGAC AAGGGCACCG GCAAGGAGAA
5851 CACGATCCGA ATCCAGGAAG GCTCGGGCCT GTCCAAGGAA GACATTGACC
5901 GCATGATCAA GGACGCCGAA GCGCACGCCG AGGAGGATCG CAAGCGTCGC
5951 GAGGAGGCCG ATGTTCGTAA TCAAGCCGAG ACATTGGTCT ACCAGACGGA
6001 GAAGTTCGTC AAAGAACAGC GTGAGGCCGA GGGTGGTTCG AAGGTACCTG
6051 AAGACACGCT GAACAAGGTT GATGCCGCGG TGGCGGAAGC GAAGGCGGCA
6101 CTTGGCGGAT CGGATATTTC GGCCATCAAG TCGGCGATGG AGAAGCTGGG
6151 CCAGGAGTCG CAGGCTCTGG GGCAAGCGAT CTACGAAGCA GCTCAGGCTG
6201 CGTCACAGGC CACTGGCGCT GCCCACCCCG GCTCGGCTGA TGAAAGCTTA
6251 AGTTTAAACC GCTAGCCTAG AGCGGCCGCG GATCCAGATC TTTTTCCCTC
6301 TGCCAAAAAT TATGGGGACA TCATGAAGCC CCTTGAGCAT CTGACTTCTG
6351 GCTAATAAAG GAAATTTATT TTCATTGCAA TAGTGTGTTG GAATTTTTTG
6401 TGTCTCTCAC TCGGAAGGAC ATATGGGAGG GCAAATCATT TAAAACATCA
6451 GAATGAGTAT TTGGTTTAGA GTTTGGCAAC ATATGCCCAT TCTTCCGCTT
6501 CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA
6551 TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA
6601 CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA
6651 AAAAGGCCGC GTTGCTGGCG TTTTTCCATA G

The plasmid pNGVL4a-SigE7(detox) HSP70-3 is schematically illustrated in Fig. 5a. A summary of this plasmid's components is shown below in Table I, as well as their position and origin.

TABLE I

Plasmid Position	Genetic Construct	Source of Construct
6491-0823	E. coli ORI (ColEI)	pBR / *E. coli* -derived
0837-0881	portion of transposase (tpnA)	Common plasmid seq, Tn5/Tn903
0882-1332	β-Lactamase (AmpR)	pBRpUC derived plasmid
1331-2496	AphA (KanR)	Tn903
2509-2691	P3 Promoter DNA binding site	Tn3/pBR322
2692-2926	pUC backbone	Common plasmid seq. pBR322-derived
2931-4009	NF1 binding and promoter	HHV-5(HCMV UL-10 IE1 gene)
4010-4044	Poly-cloning site	Common plasmid seq, pBlueScript (?)
4055-4144	Signal Peptide (Sig)	Mammalian lysosomal membrane glycoprotein A
4145-4432	dE7 gene (detoxified partial)	HPV-16 (E7 gene)
4437-6243	DNA K gene	*M. tuberculosis* HSP70
6243-6289	Poly-cloning site	Common plasmid sequence
6289-6493	Poly-Adenylation site	Mammalian signal, pHCMV-derived

A portion of SEQ ID NO:13 above vector showing by annotation the Sig, E7 (detox) and HSP-70 regions is shown below . The vector sequence is in lower case; the signal peptide (Sig) is bold italic and annotated over the lines. The E7(detox) sequence is upper case underscored (and annotated over the lines). The HSP70 sequence is italicized and underscored. (not bolded) and is also annotated over the lines

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3951 aatagctgac agactaacag actgttcctt tccatgggtc ttttctgcag
4001 tcaccgtcgt cgacggtatc gataagcttg atatcgaatt cctcgacgga
        <-----------------Signal Peptide--------------------
4051 tcttATGGCG GCCCCCGGCG CCCGGCGGCC GCTGCTCCTC CTGCTGCTGG
     ------------------------------------------------><-----
4101 CAGGCCTTGC ACATGGCGCC TCAGCACTCT TTGAGGATCT AATCATGCAT
     -----------E7(detox)-----------------------------------
4151 GGAGATACAC CTACATTGCA TGAATATATG TTAGATTTGC AACCAGAGAC
4201 AACTGATCTC TACGGTTATG GGCAATTAAA TGACAGCTCA GAGGAGGAGG
4251 ATGAAATAGA TGGTCCAGCT GGACAAGCAG AACCGGACAG AGCCCATTAC
4301 AATATTGTAA CCTTTTGTTG CAAGTGTGAC TCTACGCTTC GGTTGTGCGT
4351 ACAAAGCACA CACGTAGACA TTCGTACTTT GGAAGACCTG TTAATGGGCA
     ----------------------------------->      <-----------
4401 ACACTAGGAAT TGTGTGCCCC ATCTGTTCTC AAggatccAT GGCTCGTGCG
     -----------HSP70 (E. coli DNA K)-----------------------...
4451 GTCGGGATCG ACCTCGGGAC CACCAACTCC GTCGTCTCGG TTCTGGAAGG
4501 TGGCGACCCG CTCGTCGTCG CCAACTCCGA GGGCTCCAGG ACCACCCCGT
4551 CAATTGTCGC GTTCGCCCGC AACGGTCAGG TGCTCGTCGG CCAGCCCGCC
4601 AAGAACCAGG CGGTGACCAA CGTCGATCGC ACCGTGCGCT CGGTCAAGCG
4651 ACACATGGGC AGCGACTGGT CCATAGAGAT TGACGGCAAG AAATACACCG
4701 CGCCGGAGAT CAGCGCCCGC ATTCTGATGA AGCTGAAGCG CGACGCCGAG
4751 GCCTACCTCG GTGACGACAT TACCGACGCG GTTATCACGA CGCCCGCCTA
4801 CTTCAATG%C GCCCAGCGTC AGGCCACCAA GGACGCCGGC CAGATCGCCG
4851 GCCTCAACGT GCTGCGGATC GTCAACGAGC CGACCGCGGC CGCGCTGGCC
4901 TACGGCCTCG ACAAGCGCGA GAAGGAGCAG CCAATCCTGG TCTTCGACTT
4951 GGGTGCTGGC ACTTTCGACG TTTCCCTGCT GGAGATCGGC GAGGGTGTGG
5001 TTCAGGTCCG TGCCACTTCG GGTGACAACC ACCTCGGCGG CGACGACTGG
5051 CACCAGCGGG TCGTCGATTG GCTGGTGGAC AAGTTCAAGG GCACCAGCGG
5101 CATCGATCTG ACCAAGGACA AGATGGCGAT GCAGCGGCTG CGGGAAGCCG
5151 CCGAGAAGGC AAAGATCGAG CTGAGTTCGA GTCAGTCCAC CTCGATCAAC
5201 CTGCCCTACA TCACCGTCGA CGCCGACAAG AACCCGTTGT TCTTAGACGA
5251 GCAGCTGACC CGCGCGGAGT TCCAACGGAT CACTCAGGAG CTGCTGGACC
5301 GCACTCGCAA GCCGTTCCAG TCGGTGATCG CTGACACCGG CATTTCGGTG
5351 TCGGAGATCG ATCACGTTGT GCTCGTGGGT GGTTCGACCC GGATGCCCGC
5401 GGTGACCGAT CTGGTCAAGG AACTCACCCG CGGCAAGCAA CCCAACAAGG
5451 GCGTCAACCC CGATGACGTT GTCGCGGTGG GAGCCGCTCT GCAGGCCGGC
5501 GTCCTCAAGG GCGAGGTGAA AGACGTTCTG CTGCTTGATG TTACCCCGCT
5551 GAGCCTGGGT ATCGAGACCA AGGGCGGGGT GATGACCAGG CTCATCGAGC
5601 GCAACACCAC GATCCCCACC AAGCGGTCGG AGACTTTCAC CACCGCCGAC
5651 GACAACCAAC CGTCGGTGCA GATCCAGGTC TATCAGGGGG AGCGTGAGAT
5701 CGCCGCGCAC AACAAGTTGC TCGGGTCCTT CGAGCTGACC GGCATCCCGC
5751 CGGCGCCGCG GGGGATTCCG CAGATCGAGG TCACTTTCGA CATCGACGCC
5801 AACGGCATTG TGCACGTCAC CGCCAAGGAC AAGGGCACCG GCAAGGAGAA
5851 CACGATCCGA ATCCAGGAAG CCTCGGGCCT GTCCAAGGAA GACATTGACC
5901 GCATGATCAA GGACGCCGAA GCGCACGCCG AGGACGATCG CAAGCGTCGC
5951 GAGGAGGCCG ATGTTCGTAA TCAAGCCGAG ACATTGGTCT ACCAGACGGA
6001 GAAGTTCGTC AAAGAACAGC GTGAGGCCGA GGGTGGTTCG AAGGTACCTG
6051 AAGACACGCT GAACAAGGTT GATGCCGCGG TGGCGGAAGC GAAGGCGGCA
6101 CTTGGCGGAT CGGATATTTC GCCCATCAAG TCGGCGATGG AGAAGCTGGG
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6151 CCAGGAGTCG CAGGCTCTGG GGCAAGCGAT CTACGAAGCA GCTCAGGCTG

     ------------------------------------------->
6201 CGTCACAGGC CACTGGCGCT GCCCACCCCG GCTCGGCTGA TGAaagctta
6251 agtttaaacc gctagcctag agcggccgcg gatccagatc tttttccctc
6301 tgccaaaaat tatggggaca tcatgaaccc ccttgagcat ctgacttctg
...
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GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al. Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press, Berkeley, CA (1981).

Techniques for the manipulation of nucleic acids, such as, *e.g.*, generating mutations in sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature. See, *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Tijssen, ed. Elsevier, N.Y. (1993).

Nucleic acids, vectors, capsids, polypeptides, and the like can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyper diffusion chromatography, various immunological methods, *e.g.* fluid or gel precipitin reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (Rios), enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (*e.g.*, SDS-PAGE), RTF-PCR,

quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Amplification of Nucleic Acids

Oligonucleotide primers can be used to amplify nucleic acids to generate fusion protein coding sequences used to practice the invention, to monitor levels of vaccine after *in vivo* administration (*e.g.*, levels of a plasmid or virus), to confirm the presence and phenotype of activated CTLs, and the like. The skilled artisan can select and design suitable oligonucleotide amplification primers using known sequences. Amplification methods are also well known in the art, and include, *e.g.*, polymerase chain reaction, PCR (*PCR Protocols, A Guide to Methods and Applications*, Academic Press, N.Y. (1990) and *PCR Strategies* (1995), Academic Press, Inc., N.Y., ligase chain reaction (LCR) (Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained sequence replication (Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Qβ replicase amplification (Smith (1997) J. Clin. Microbiol. 35:1477-1491; Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (NASBA, Cangene, Mississauga, Ontario; Berger (1987) Methods Enzymol. 152:307-316; U.S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995) Biotechnology 13:563-564).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be

synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a translocation polypeptide and an antigen, fragments thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism in a nucleotide sequence encoding an anti-apoptotic polypeptide according to the present invention (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family members are defined as proteins that share function amino acid sequence similarity to the reference polypeptide.

Fragment of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length translocation polypeptide, antigenic polypeptide or the fusion thereof.. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen part of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes an anti-apoptotic polypeptide that retains the ability to improve the immunogenicity of an antigenic composition

when administered as a chimeric DNA with antigen-encoding sequence, or when co-administered therewith.

Generally, the nucleic acid sequence encoding a fragment of an anti-apoptotic polypeptide comprises of nucleotides from the sequence encoding the mature protein (or an active fragment thereof).

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for translocation types of proteins, and DNA coding sequences for antigenic polypeptides, include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a anti-apoptotic polypeptide or a targeting polypeptide operably linked to at least one regulatory sequence. These vectors are also in the process of producing the final vaccine vector.

The term "expression vector" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology,* vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (*e.g.*, a cell membrane, a viral lipid envelope, *etc.*). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.*

Such expression vectors are used to transfect host cells (*in vitro*, *ex vivo* or *in vivo*) for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose. Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the fusion polypeptide and DNA encoding at least a portion of a second protein, so that the host cells produce yet further fusion polypeptides that include both the portions. A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology,* 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, such as a nucleic acid, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, *etc.*) or from other contaminants with which it is natively associated or becomes associated during processing.. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques .

Prokaryotic or eukaryotic host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

Vector Construction

Construction of suitable vectors comprising the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Promoters and Enhancers

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions encountered in the cell's environmental

and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No. 5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

Nucleic acids of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known,

including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

PROTEINS AND POLYPEPTIDES

The terms "polypeptide," "protein," and "peptide" when referring to compositions of the invention are meant to include variants, analogues, and mimetics with structures and/or activity that substantially correspond to the polypeptide or peptide from which the variant, *etc.*, was derived.

The present invention includes the expression *in vivo* of fusion polypeptides comprising a targeting polypeptide linked to an antigenic polypeptide.

The term "chimeric" or "fusion" polypeptide or protein refers to a composition comprising at least one polypeptide or peptide sequence or domain that is chemically bound in a linear fashion with a second polypeptide or peptide domain. One embodiment of this invention is an isolated or recombinant nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises an anti-apoptotic polypeptide and the second domain comprising an antigenic epitope, *e.g.*, an MHC class I-binding peptide epitope. Additional domains can comprise a targeting polypeptide or the like. The "fusion" can be an association generated by a peptide bond, a chemical linking, a charge interaction (*e.g.*, electrostatic attractions, such as salt bridges, H-bonding, *etc.*) or the like. If the polypeptides are recombinant, the "fusion protein" can be translated from a common mRNA. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention (*e.g.*, targeting polypeptide fusion proteins) can also include additional sequences, *e.g.*, linkers, epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the fusion polypeptide, or a cell expressing this nucleic acid is administered to a mammalian subject, preferably a human. The vaccine composition is administered in a pharmaceutically acceptable carrier in a biologically effective or a therapeutically effective amount, Certain preferred conditions are

disclosed in the Examples. The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount is between about 1 nanogram and about 1 gram per kilogram of body weight of the recipient, more preferably between about 0.1 μg/kg and about 10mg/kg, more preferably between about 1 μg/kg and about 1 mg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.1 μg to 100 μg of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of cells expressing the nucleic acid is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The active compound may be administered in a convenient manner, *e.g.*, injection by a convenient and effective route. Preferred routes include intradermal "gene gun" delivery, and intramuscular routes. For the treatment of existing tumors which have not been completely resected or which have recurred, direct intratumoral injection may also be used.

Depending on the route of administration, the nucleic acid may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme

inhibitors of nucleases or proteases or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol* 7:27).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material

(*e.g.*, the nucleic acid vaccine) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

Antigens Associated with Pathogens

A major use for the present invention is the use of the present nucleic acid compositions in therapeutic vaccine for cancer and for major chronic viral infections that cause morbidity and mortality worldwide. Such vaccines are designed to eliminate infected cells - this requires T cell responses as antibodies are often ineffective. The vaccines of the present invention are designed to meet these needs.

Preferred antigens are epitopes of pathogenic microorganisms against which the host is defended by effector T cells responses, including cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity. These typically include viruses, intracellular parasites such as malaria, and bacteria that grow intracellularly such as Mycobacteria and Listeria species. Thus, the types of antigens included in the vaccine compositions of this invention are any of those associated with such pathogens (in addition, of course, to tumor-specific antigens). It is noteworthy that some viral antigens are also tumor antigens in the case where the virus is a causative factor in cancer.

In fact, the two most common cancers worldwide, hepatoma and cervical cancer, are associated with viral infection. Hepatitis B virus(HBV) (Beasley, R.P. *et al.*, *Lancet* **2**, 1129-1133 (1981) has been implicated as etiologic agent of hepatomas. 80-90% of cervical cancers express the E6 and E7 antigens (exemplified herein) from one of four "high risk" human papillomavirus types: HPV-16, HPV-18, HPV-31 and HPV-45 (Gissmann, L. *et al.*, *Ciba Found Symp.* **120**, 190-207 (1986); Beaudenon, S., *et al. Nature* **321**, 246-249 (1986). The HPV E6 and E7 antigens are the most promising targets for virus associated cancers in immunocompetent individuals because of their ubiquitous expression in cervical cancer. In addition to their importance as targets for therapeutic cancer vaccines, virus associated tumor antigens are also ideal candidates for prophylactic vaccines. Indeed, introduction of prophylactic HBV vaccines in Asia have decreased the incidence of hepatoma (Chang, M.H., *et al. New Engl. J. Med.* **336,** 1855-1859 (1997), representing a great impact on cancer prevention.

Among the most important viruses in chronic human viral infections are HPV, HBV, hepatitis C Virus (HCV), human immunodeficiency virus (HIV-1 and HIV-2), herpesviruses such as Epstein Barr Virus (EBV),cytomegalovirus (CMV) and HSV-1 and HSV-2 and influenza virus. Useful antigens include HBV surface antigen or HBV core antigen; ppUL83 or pp89 of CMV; antigens of gp120, gp41 or p24 proteins of HIV-1; ICP27, gD2, gB of HSV; or influenza nucleoprotein (Anthony, LS *et al., Vaccine* 1999; 17:373-83). Other antigens associated with pathogens that can be utilized as described herein are antigens of various parasites, includes malaria, preferably malaria peptide (NANP)40.

In addition to its applicability to human cancer and infectious diseases,, the present invention is also intended for use in treating animal diseases in the veterinary medicine context. Thus, the approaches described herein may be readily applied by one skilled in the art to treatment of veterinary herpesvirus infections including equine herpesviruses, bovine viruses such as bovine viral diarrhea virus (for example, the E2 antigen), bovine herpesviruses, Marek's disease virus in chickens and other fowl; animal retroviral and lentiviral diseases (*e.g.*, feline leukemia, feline immunodeficiency, simian immunodeficiency viruses, *etc.*); pseudorabies and rabies; and the like.

As for tumor antigens, any tumor-associated or tumor-specific antigen that can be recognized by T cells, preferably by CTL, can be used. In addition to the HPV-E7 antigen exemplified herein is mutant p53 or HER2/neu or a peptide thereof. Any of a number of

melanoma-associated antigens may be used, such as MAGE-1, MAGE-3, MART-1/Melan-A, tyrosinase, gp75, gp100, BAGE, GAGE-1, GAGE-2, GnT-V, and p15 (see, US 6,187,306).

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology*, Fields, BN *et al.*, eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint*, S.J. *et al.*, eds., Amer Society for Microbiology, Washington, 1999; *Principles and Practice of Clinical Virology*, 4th Edition, Zuckerman. A.J. *et al.*, eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses*, by Hagedorn, CH *et al.*, eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy,* Koshy, R. *et al.*, eds,, World Scientific Pub Co, 1998; *Veterinary Virology*, Murphy, F.A. *et al.*, eds., Academic Press, NY, 1999; *Avian Viruses: Function and Control*, Ritchie, B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses,* by M. H. V. Van Regenmortel, MHV *et al.*, eds., Academic Press; NY, 2000.

Delivery of Vaccine Nucleic Acid to Cells and Animals

The Examples below describe certain preferred approaches to delivery of the vaccines of the present invention. A broader description of other approaches including viral and nonviral vectors and delivery mechanisms follow.

DNA delivery involves introduction of a "foreign" DNA into a cell *ex vivo* and ultimately, into a live animal or directly into the animal. Several general strategies for gene delivery (= delivery of nucleic acid vectors) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460 (1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al., Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al., Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al., Cancer Invest. 7*:179-192 (1989), which references are herein incorporated by reference in their entirety).

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue

or organ *in vivo*. DNA transfer can be achieved using a number of approaches described herein. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA 87*:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 280*:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 244*:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol. 27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta 1088*:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al.*, *J. Biol. Chem. 264*:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem. 263*:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem. 267*:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA according to the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

MATERIALS AND METHODS

Mice: Six- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, Maryland) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Maryland). All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

Plasmid DNA Construction: The E7/70 gene was cloned into pNGVL4a (National Gene Vector Laboratory) using the *EcoRI* and *KpnI* restriction sites. Using site-directed mutagenesis, two point mutations, which had previously been found to reduce Rb binding (Munger, K *et al.*, *EMBO J* 1989, **8:**4099-4105), were introduced into the E7 gene. The primers used to introduce these mutations were as follows:

mE7 Forward: 5' ctgatctctacggttatgggcaattaaatgacagctc 3' [SEQ ID NO:14] and

mE7 Reverse: 5' gagctgtcatttaattgcccataaccgtagagatca 3' [SEQ ID NO:15] .

For construction of pNGVL4a-Sig/E7(detox)/HSP70, Sig was cut from pCMV-neoSig/E7/LAMP-1 (Chen, CH *et al.*, *Gene Ther* 1999, **6:**1972-1981) by EcoRI/NsiI, and E7(detox)/HSP70 was cut from pNGVL4a-E7(detox)/HSP70 by NsiI/XbaI. Sig and E7(detox)/HSP70 were ligated and cloned into pNGVL4a vector cut with EcoRI/XbaI. The accuracy of DNA constructs was confirmed by DNA sequencing.

The production and maintenance of TC-1 cells has been described previously (Lin, KY *et al.*, *Cancer Res* 1996, **56:**21-26.). In brief, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate TC-1.

Needle IM Mediated DNA Vaccination: The pNGVL4a-Sig/E7(detox)/HSP70 vaccine was administered to groups of mice by three different methods. For needle i.m. mediated DNA vaccination, 50μg/mouse of pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccines were delivered intramuscularly by syringe needle injection. The dose was chosen based on the optimal dosage conditions most commonly used in other studies [Gurunathan, S *et al.*, , *Annu Rev Immunol* 2000, **18:**927-974]. These mice received a booster with the same regimen one week later.

Biojector Mediated DNA Vaccination: The Biojector 2000 (Bioject Inc., Portland, OR) is a needle-free jet injection device consisting of an injector and a disposable syringe. The orifice size controls the depth of penetration. 50μg/mouse of pNGVL4a-Sig/E7(detox)/HSP70 were delivered to the shaved flank region of C57BL/6 mice using the Biojector with no. 2 syringe nozzle. We have adopted the same dosage used in needle i.m. for the biojector administrations. These mice received a booster with the same regimen one week later.

Gene Gun Mediated DNA Vaccination: DNA-coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of C57BL/6 mice using a helium-driven gene gun (BioRad, Hercules, CA) with a discharge pressure of 400 p.s.i. C57BL/6 Mice were vaccinated via gene gun with 2 μg of pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine. The dose was chosen based on the optimal dosage conditions most commonly used in other studies (Gurunathan et al., *supra*). These mice received a booster with the same regimen one week later.

Intracellular Cytokine Staining with Flow Cytometry Analysis to Detect IFN-γ Secretion by E7-Specific $CD8^+$ T Cells: Cell surface marker staining for CD8 and intracellular cytokine staining for IFN-γ as well as FACScan analysis were performed using conditions described previously (Chen, CH et al., *Vaccine* 2000, **18:**2015-2022). Prior to FACScan, splenocytes from different vaccinated groups of mice were collected and incubated for 20 hours either with or without 1 μg/ml of E7 peptide (aa 49-57, RAHYNIVTF (SEQ ID NO:16) [Feltkamp, MC et al., *Eur J Immunol* 1993, **23:**2242-2249] containing an MHC class I epitope for detecting E7-specific CD8 T cell precursors. The number of IFN-γ-secreting $CD8^+$ T cells was analyzed using flow cytometry. Analysis was performed on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

***In vivo* Tumor Treatment Experiment :** An *in vivo* tumor treatment experiment was performed using the conditions described previously (Ji, H *et al.*, Int J Cancer 1998, 78:41-45]. Mice (5 per group) were intravenously challenged with 1x10^4 cells/mouse TC-1 tumor cells via tail vein. Three days after tumor challenge, mice were administered 50μg/mouse of DNA vaccine by i.m. or biojector or were administered 2μg/mouse of DNA vaccine by gene gun. One week after the first vaccination, these mice were boosted with the same regimen and method as the first vaccination. Mice were sacrificed and lungs were explanted on day 28 after TC-1 challenge. The number of pulmonary tumor nodules and the total pulmonary weight in each mouse were evaluated.

***In Vivo* Tumor Protection Experiment:** An *in vivo* tumor protection experiment was performed using the conditions described previously [Lin et al., *supra*]. Mice (5 per group) received 50μg/mouse of DNA vaccines by i.m. or biojector, or mice received 2μg/mouse by gene gun. One week later, mice were boosted with the same regimen and method as the first vaccination. Mice were subcutaneously challenged with 5x10^4 cells/mouse TC-1 tumor cells one week after the last vaccination. Tumor growth was examined by palpation and inspection twice a week. Percentage of tumor free mice were recorded.

Statistical Analysis: All data expressed as means ± SE are representative of at least two different experiments. Data for intracellular cytokine staining with flow cytometry analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using a student's t-test. In the tumor protection experiment, the principal outcome of interest was time to development of tumor. The event time distributions for different mice were compared by use of the method of Kaplan and Meier and by use of the log-

EXAMPLE II

pNGVL4a-Sig/E7(detox)/HSP70 DNA Vector Administered by Gene Gun Generated Highest Number of E7-specific CD8$^+$ T cells in Immunized Mice

This study compared the ability of the pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine composition to generate E7-specific CD8$^+$ T cell precursors by evaluation intracellular cytokines in splenocytes from mice vaccinated with the same DNA construct by three different modes of administration: needle i.m., biojector, and gene gun. Splenocytes from naïve or immunized

vaccinated groups of mice were incubated with or without the MHC class I (H-2 D^b)-restricted E7 peptide (aa 49-57) (SEQ ID NO:2) to detect E7-specific $CD8^+$ T cells.

Results are shown in **Figures 1A and 1B/** Mice vaccinated via gene gun exhibited significantly higher numbers ($p<0.05$) of E7-specific IFN-γ^+ $CD8^+$ T cell precursors per fixed number of splenocyte (832.5) compared to mice vaccinated via biojector (445.5) and needle i.m. (375.5). These findings suggest that the gene gun approach was somewhat more potent in this setting. However, i.m. injection was also effective.

EXAMPLE III

Vaccinated Mice were Protected *In Vivo* against E7-expressing Tumors

The next study investigated protection against TC-1 tumor, expressing the same antigen, E7, as the pNGVL4a-Sig/E7(detox)/HSP70 vaccine, administered by the three routes. *In vivo* tumor protection experiment (vaccination before tumor challenge) used the well-characterized E7-expressing tumor model, TC-1. As shown in **Figure 2**, mice receiving pNGVL4a-Sig/E7(detox)/HSP70 via gene gun, biojector, or needle i.m. remained 100% tumor free after TC-1 tumor challenge. Thus, this vaccine vector administered by different routes generated total protection against growth of later-administered tumor cells expressing the E7 antigen.

EXAMPLE IV

The E7 Vaccine was Therapeutic in mice bearing the TC-1 tumor

Sig/E7(detox)/HSP70 vaccine was administered to mice bearing the TC-1 tumor under conditions where, in untreated controls, tumor metastasis to the lungs occurred by hematogenous spread. Metastasis was assessed by enumeration of lung nodules and by weighing lungs. As shown in **Figures 3 and 4**, mice receiving pNGVL4a-Sig/E7(detox)/HSP70 via gene gun exhibited the lowest number of pulmonary nodules and lowest pulmonary weight of the three groups of vaccinated mice and naïve controls. The differences between the three vaccinated groups did not reach statistical significance.

Furthermore, the variance (standard deviation and standard error (SE)) in the number of pulmonary nodules and pulmonary weight was smaller in the animals vaccinated by gene gun and biojector as compared to the groups vaccinated by injection i.m. with a syringe.

DISCUSSION of EXAMPLES I-IV

The foregoing studies focused on the immune and anti-tumor responses of mice immunized with pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine administered via needle i.m., biojector, and gene gun in mice. The studies employed doses and conditions shown by the present inventors and their colleagues to be optimal

The results indicated that the DNA vaccine administered via gene gun generated the highest immune response, measured as the number of E7-specific $CD8^+$ T cells. The efficacy of this approach was further supported by the fact that gene gun immunization required the least amount of the immunogen to generate a similar or slightly higher anti-tumor effects.

These observed differences may be attributable to the capacity of these routes of administration to generate professional antigen presenting cells (APCs) that express the antigen. Intradermal immunization by gene gun can directly target antigen to the skin's professional APCs - , Langerhans cells (Condon, C et al., *Nat Med* 1996, **2:**1122-1128; Porgador, A *et al.*, *J Exp Med* 1998, **188:**1075-1082), allowing improved direct presentation of antigen to T cells by DNA-transfected DCs. In comparison, intramuscular immunization likely targets antigen to myocytes and the antigen encoded by the DNA vaccine is eventually is presented through bone marrow-derived APCs [Fu, TM et al., *Mol Med* 1997, **3**(6), 362-37119]. In this setting, the number of professional APCs expressing antigen may be lower. In these ways, it appears that the route of administration may influence the ability of the present inventors' strategy to enhance DNA vaccine potency.

Although gene gun-mediated vaccination may have generated the highest number of antigen-specific $CD8^+$ T cells, mice receiving the DNA vector via all three routes were protected, remaining 100% tumor free, after TC-1 tumor challenge **(Figure 2)**. It may be that that the experimental window (*i.e.*, time frame of observation and size of tumor inoculum) may not have been sufficient to allow a clear distinction among the three routes.

However, using a more stringent tumor treatment model, only slight and statistically insignificant differences were observed in the number of pulmonary nodules and pulmonary weight among the mice vaccinated with syringe, biojector, and gene gun.

CD8+ T cells are clearly important for the observed antitumor effects of the pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine.

Using human papillomavirus type 16 E7 as a model antigen, the present inventors and colleagues previously evaluated the impact of linking antigen-encoding DNA to DNA encoding HSP70 on the potency of antigen-specific immunity generated by naked DNA vaccines . Vaccines comprising E7-HSP70 fusion genes increased the frequency of E7-specific CD8+ T cells relative to vaccines containing the wild-type E7 gene alone. More importantly, *in vivo* antibody depletion experiment demonstrated that E7-HSP70 fusion vaccines exclusively targeted CD8+ T cells; immunological and antitumor effects were completely independent of CD4+ T cells and NK cells.

The addition of a signal peptide (Sig) to E7/HSP70 may lead to exogenous release of chimeric E7/HSP70 protein. Suto *et al. Science* 1995, **269:**1585-1588, demonstrated that an exogenous antigen chaperoned by a heat shock protein can be channeled into the endogenous pathway, presented by MHC class I molecules, and recognized by CD8+ T lymphocytes in the classical phenomenon known as "cross-priming." Additionally, *in vivo* studies by Chu *et. al.*, *Clin Exp Immunol* 2000, **121:**216-225, using mice depleted of CD8 or CD4 lymphocyte subsets demonstrated that tumor regression following therapeutic hspE7 protein immunization was CD8-dependent and CD4-independent. Moreover, Huang *et al. J Exp Med* 2000, **191:**403-408, showed that Hsp70 fusion proteins elicit CD4-independent CTL responses. Thus, there are several bases for the conclusion that fusion of HSP70 to E7 enhances the potency of vaccines via CD8-dependent pathways.

The administration of the pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine via the gene gun and biojector may provide more consistency (*i.e.*, lower variance) than intramuscular administration via syringe. Results appearing in Figure 3 and 4 showed that the variance in the number of pulmonary nodules and pulmonary weight was smaller when the DNA vaccine is administered by gene gun and biojector than by syringe.

Thus, it appears that the instrumentation and method of delivery may influence the consistency of response to this vaccine. Both the gene gun apparatus and the biojector device utilize a standard mechanical method of delivering DNA and may avoid some of the inconsistency introduced by a method that is inherently more variable. Results of previous

studies suggested that the biojector apparatus may be a more consistent means than a syringe in delivering such vaccines (Lemon, SM *et al.*, *J Med Virol* 1983, 12:129-136; Aguiar, JC *et al.*, *Vaccine* 2001, 20:275-280; Rogers, WO *et al.*, *Infect Immun* 2001, 69:5565-5572).

For mass immunization of humans, safety is an important consideration. First, the DNA may integrate into the host genome, resulting in the inactivation of tumor suppresser genes or the activation of oncogenes which could lead to malignant transformation of the host cell. Because it is estimated that the frequency of integration is much lower than that of spontaneous mutation, integration should not pose any real risk (Nichols, WW et al., *Ann N Y Acad Sci* 1995, 772:30-39). A second concerns is the potential risk associated with the presence of HPV-16 E7 oncoprotein in host cells. The same concern holds true for the HPV E6 protein. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei [Lukas, J, *J Cell Biol* 1994, 125:625-638] . The presence of E7 in host cells may lead to an accumulation of genetic aberrations and eventual malignant transformation. The pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine allays the latter concern by introducing two key mutations into E7 which preserve its immunogenicity but alleviate its oncogenicity by destroying its ability to bind pRB. A third concern is the risk of inducing for autoimmunity mediated by CTL clones specific for mycobacterial HSP that might cross-react to host HSP. Pathological examination of the vital organs in the pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccinated mice (and recipients of all other similar DNA vaccines that the present inventors have tested) did not reveal any detrimental or other autoimmune pathology.

In summary, as shown herein, vaccination with pNGVL4a-Sig/E7(detox)/HSP70 DNA is a safe and effective way to enhance antigen-specific $CD8^+$ T cell immune responses and antigen-specific antitumor effects.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not. All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

Citation of the documents herein is not intended as an admission that any of them is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

CLAIMS

1. A nucleic acid molecule encoding a fusion polypeptide useful as a vaccine composition, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide or peptide that promotes processing via the MHC class I pathway;

(b) fused in frame with the first nucleic acid sequence, a second nucleic acid sequence encoding a signal peptide; and

(c) a third nucleic acid sequence that is linked in frame to said first nucleic acid sequence and that encodes an antigenic polypeptide or peptide.

2. The nucleic acid molecule of claim 1, wherein the antigenic peptide comprises an epitope that binds to a MHC class I protein.

3.. The nucleic acid molecule of claim 1 wherein the first polypeptide or peptide is Hsp70, an active C-terminal domain thereof, or a functional derivative of Hsp70 or of said C-terminal domain.

4. The nucleic acid molecule of claim 1, wherein the first polypeptide is encoded by SEQ ID NO:9 or a fragment thereof that encodes a functional derivative of said polypeptide or the full length sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

5. The nucleic acid molecule of claim 1, wherein the first polypeptide is SEQ ID NO:10 of a functional derivative thereof.

6. The nucleic acid molecule of claim 1 wherein the antigen is one which is present on, or/ cross-reactive with an epitope of, a pathogenic organism, cell, or virus.

7. The nucleic acid molecule of claim 6, wherein the virus is a human papilloma virus.

8. The nucleic acid molecule of claim 7, wherein the antigen is an E7 polypeptide of HPV-16 having the sequence SEQ ID NO:2, or an antigenic fragment thereof.

9. The nucleic acid molecule of claim 8, wherein the HPV-16 E7 polypeptide is a non-oncogenic mutant or variant of said E7 polypeptide.

10. The non oncogenic mutant of claim 9 wherein the sequence of the E7 polypeptide differs from SEQ ID NO:2 by one or more of the following substitutions:

(a) Cys at position 24 to Gly or Ala

(b) Glu at position 26 to Gly or Ala

(c) Cys at position 91 to Gly or Ala.

11. The nucleic acid molecule of claim 7, wherein the antigen is the E6 polypeptide of HPV-16 having the sequence SEQ ID NO:4 or an antigenic fragment thereof.

12. The nucleic acid molecule of claim 11, wherein the HPV-16 E6 polypeptide is a non-oncogenic mutant or variant of said E6 polypeptide.

13. The non oncogenic mutant of claim 12 wherein the sequence of the E6 polypeptide differs from SEQ ID NO:4 by one or more of the following substitutions:

(a) Cys at position 70 to Gly or Ala

(b) Cys at position 113 to Gly or Ala.

(c) Ile at position 135 to Thr

14. The nucleic acid molecule of claim 1 that is characterized as pNGVL4a-Sig/E7(detox)/HSP70, and has the sequence SEQ ID NO:13.

15. The nucleic acid molecule of claim 1 operatively linked to a promoter.

16. An expression vector comprising the nucleic acid molecule of any of claims 1-13 operatively linked to

(a) a promoter; and

(b) optionally, additional regulatory sequences that regulate expression of said nucleic acid in a eukaryotic cell.

17. An expression vector comprising the nucleic acid molecule of claim 14. operatively linked to

(a) a promoter; and

(b) optionally, additional regulatory sequences that regulate expression of said nucleic acid in a eukaryotic cell.

18. The expression vector of claim 16 which is a plasmid.

19. The expression vector of claim 18 wherein said plasmid is pNGV4a.

20. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition comprising the nucleic acid molecule of any of claims 1-13.

21. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the nucleic acid molecule of claim 14.

22. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the expression vector of claim 16.

23. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the expression vector of claim 19.

24. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 20, thereby inducing or enhancing said response.

25. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 21, thereby inducing or enhancing said response.

26. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 22, thereby inducing or enhancing said response.

27. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 23, thereby inducing or enhancing said response.

28. The method of claim 24, wherein the response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

29. The method of claim 24 wherein said subject is a human.

30. The method of claim 25 wherein said subject is a human.

31. The method of claim 26 wherein said subject is a human.

32. The method of claim 27 wherein said subject is a human.

33. The method of claim 29 wherein said administering is by a intramuscular injection by gene gun administration or by needle-free jet injection.

34. The method of claim 30 wherein said administering is by a intramuscular injection by gene gun administration or by needle-free jet injection.

35. The method of claim 31 wherein said administering is by a intramuscular injection by gene gun administration or by needle-free jet injection.

36. The method of claim 32 wherein said administering is by a intramuscular injection by gene gun administration or by needle-free jet injection.

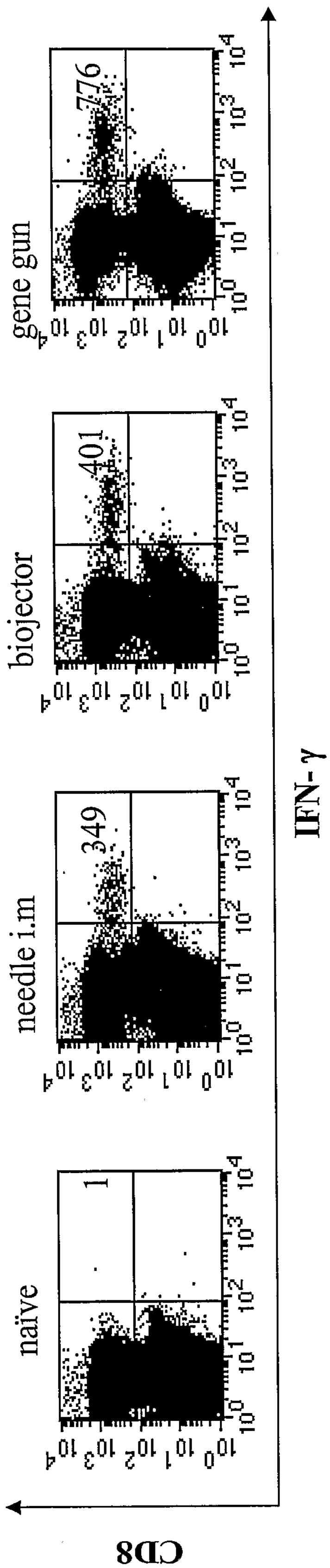
37. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 20, wherein said third nucleic acid sequence encodes one or more epitopes of E7 or E6, thereby inhibiting said growth or preventing said re-growth.

38. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 21, wherein said third nucleic acid sequence encodes one or more epitopes of E7 or E6, thereby inhibiting said growth or preventing said re-growth.

39. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 22, wherein said third nucleic acid sequence encodes one or more epitopes of E7 or E6, thereby inhibiting said growth or preventing said re-growth.

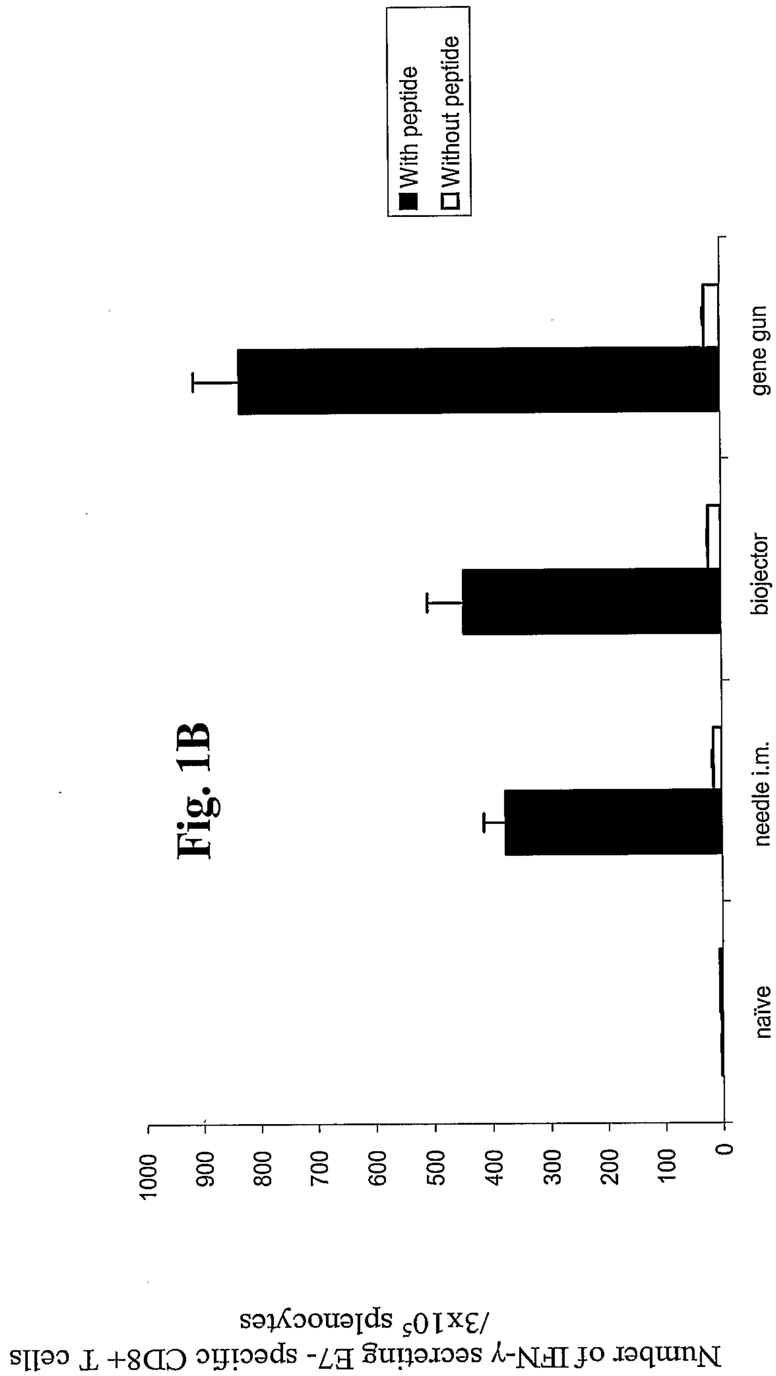
40. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 23, wherein said third nucleic acid sequence encodes one or more epitopes of E7 or E6, thereby inhibiting said growth or preventing said re-growth.

Fig. 1A



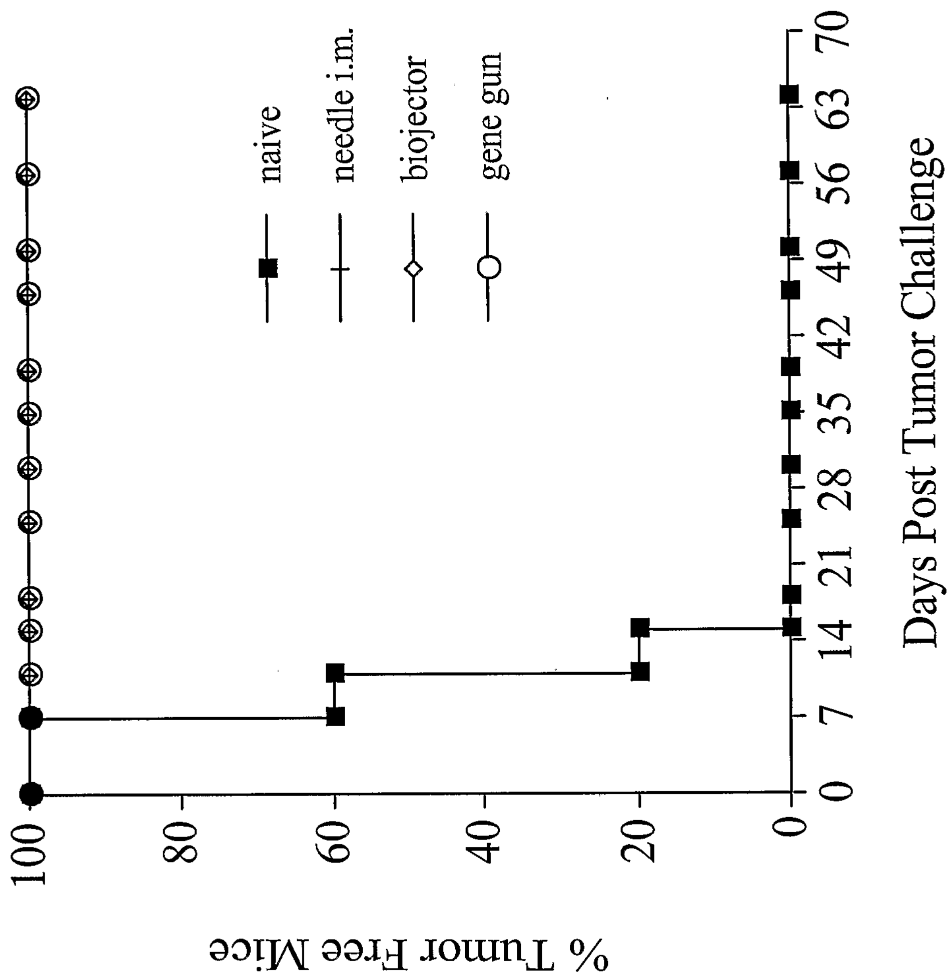
naïve
needle i.m
biojector
gene gun
1
349
401
776
CD8
IFN-γ

Fig. 1B



Number of IFN-γ secreting E7- specific CD8+ T cells /3x10⁵ splenocytes
With peptide
Without peptide
naïve
needle i.m.
biojector
gene gun

Fig. 2


% Tumor Free Mice
100
80
60
40
20
0
0
7
14
21
28
35
42
49
56
63
70
Days Post Tumor Challenge
naive
needle i.m.
biojector
gene gun

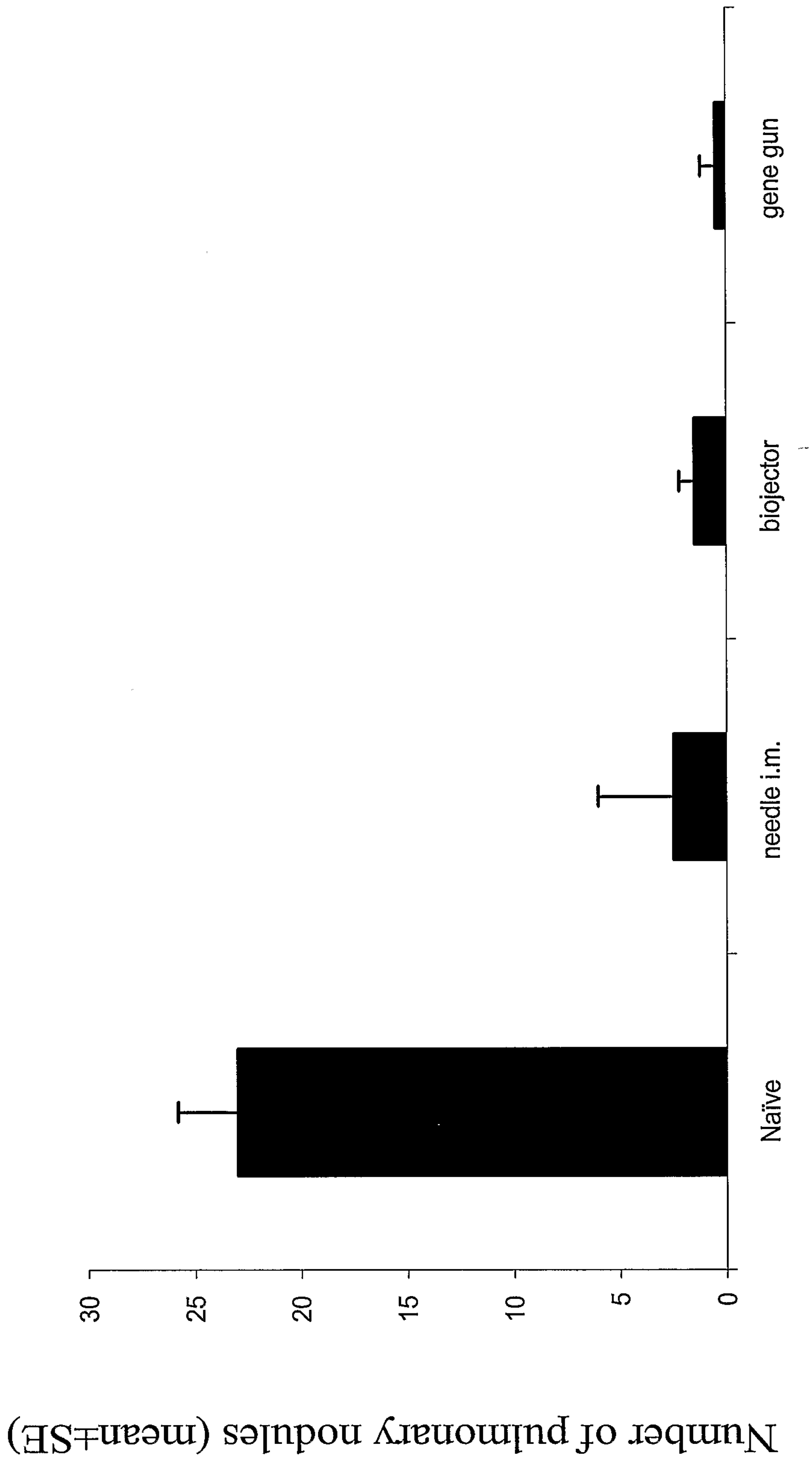

Number of pulmonary nodules (mean±SE)
0
5
10
15
20
25
30
Naïve
needle i.m.
biojector
gene gun

Fig. 3

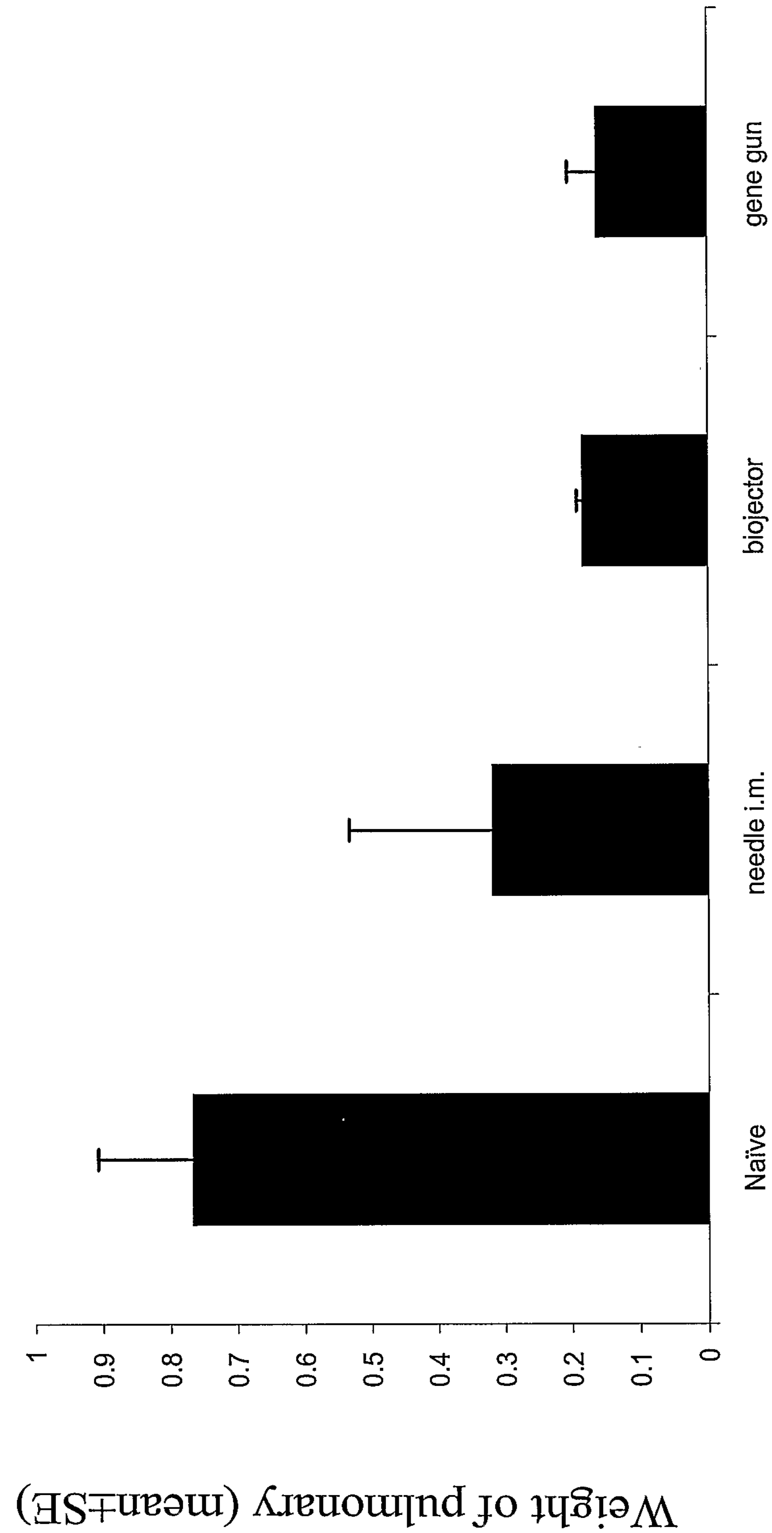
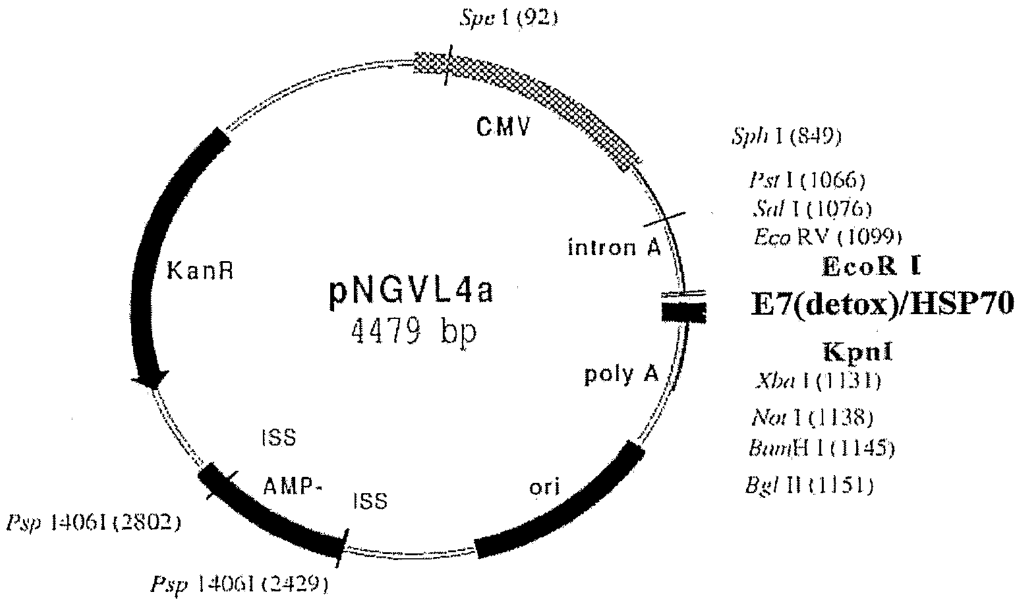

Weight of pulmonary (mean±SE)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Naïve
needle i.m.
biojector
gene gun

Fig. 4

Fig. 5A



Point mutations introduced to generate detoxified E7/hsp70

Fig. 5B

```
                pos 24    pos 26
     L   Y   C   Y   E   Q   L
....CTC TAC TGT TAT GAG CAA TTA....

     L   Y   G   Y   G   Q   L
....CTC TAC GGT TAT GGG CAA TTA....
```